

PCT

BEST AVAILABLE COPY

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 38/17, C07K 14/875</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/53846</b> <b>(43) International Publication Date:</b> 3 December 1998 (03.12.98)
<b>(21) International Application Number:</b> PCT/US98/11026 <b>(22) International Filing Date:</b> 28 May 1998 (28.05.98)  <b>(30) Priority Data:</b> 08/864,357 28 May 1997 (28.05.97) US  <b>(71) Applicants:</b> CLARAGEN, INC. [US/US]; 335 Paint Branch Road, College Park, MD 20742 (US). NATIONAL INSTITUTE OF HEALTH [US/US]; Dept. of Health and Human Services, Office of Technology Transfer, Suite 325, 6011 Executive Building, Rockville, MD 20852 (US).  <b>(72) Inventors:</b> PILON, Aprile, L.; 11333 Rambling Road, Gaithersburg, MD 20879 (US). MUKHERJEE, Anil, B.; P.O. Box 136, Brookeville, MD 20833-0136 (US). ZHANG, Zhongjian; 10 Chantilly Court, Rockville, MD 20850 (US).  <b>(74) Agents:</b> EVANS, Barry et al.; Whitman Breed Abbott & Morgan LLP, 200 Park Avenue, New York, NY 10166 (US).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> USE OF RECOMBINANT HUMAN UTEROGLOBIN IN TREATMENT OF INFLAMMATORY AND FIBROTIC CONDITIONS  <b>(57) Abstract</b>  Methods for treatment of inflammatory and fibrotic conditions <i>in vivo</i> using UG is disclosed. Methods for treating or preventing inflammatory or fibrotic conditions characterized by a deficiency of endogenous functional UG are also disclosed. Compositions containing UG, optionally containing lung surfactant, and assay procedures for detection of UG-fibronectin complexes, are also provided.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## USE OF RECOMBINANT HUMAN UTEROGLOBIN IN TREATMENT OF INFLAMMATORY AND FIBROTIC CONDITIONS

### CROSS-REFERENCE TO RELATED APPLICATIONS

5

The present application is a continuation-in-part of U.S. Application Serial No. 08/864,357, the disclosure of which is incorporated herein by reference.

### FIELD OF THE INVENTION

10

The invention relates generally to the treatment of inflammatory and fibrotic conditions using native human uteroglobin (hUG) or recombinant human uteroglobin (rhUG). Novel physiological roles and therapies for UG (hUG or rhUG) have been identified. Specifically, the invention relates to the treatment of inflammatory and  
15 fibrotic conditions by administering hUG or rhUG to inhibit PLA<sub>2</sub>s and/or to prevent fibronectin deposition. The invention further provides a method for the treatment of neonatal respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD), a critical clinical condition of the lung, and glomerular nephropathy, a disease of the kidney, both characterized by the inflammatory and fibrotic conditions.

20

Documents cited in this application relate to the state-of-the-art to which this invention pertains, each of which is incorporated herein by reference.

### BACKGROUND OF THE INVENTION

#### Inflammatory and Fibrotic Conditions

25

The search for improved therapeutic agents for the treatment of inflammatory and fibrotic diseases has received much attention in recent years. Neonatal RDS, a lung surfactant deficiency disease, is a condition of particular interest in that it is one of the major causes of mortality in premature neonates. While introduction of surfactant therapy dramatically improves survival of RDS patients, the development  
30 of chronic inflammatory and fibrotic disease in a significant percentage of this patient population is a major problem. Likewise, hereditary fibronectin-deposit glomerular nephropathy leads to end stage renal failure when patients' kidneys become blocked

and no longer filter the blood. Nephropathy is characterized by fibronectin deposits and fibrosis of the kidneys which render the organ non-functional, and eventually, unable to support life.

PLA<sub>2</sub> (phospholipase A<sub>2</sub>), a class of endogenous enzymes that hydrolyze the  
5 Sn2 position ester bond of glycerophospholipids, is one of many proteins implicated in inflammatory and fibrotic conditions. It is also responsible for hydrolysis of surfactant phospholipids in the lungs. Uteroglobin (also known as CC10, CC16, CC17, urine protein-1, P-1, progesterone binding protein, PCB-binding protein, Clara cell secretory protein (CCSP), blastokinin, retinol-binding protein, phospholipid-  
10 binding protein, and alpha2-microglobulin) inhibits the activity of PLA<sub>2</sub> *in vitro*.

Uteroglobin is a small globular homodimeric protein. It has a molecular weight of 15.8 kDa, but it migrates in electrophoretic gels at a size corresponding to 10 kDa. Human uteroglobin is abundant in the adult human lung, and comprises up to about 7% of the total soluble protein. However, its expression is not fully  
15 activated in the developing human fetus until late in gestation. Consequently, the extracellular lung fluids of pre-term infants contain far less UG than those of adults. UG is also expressed by the pancreas.

PLA<sub>2</sub>s play critical roles in the inflammatory response because they release arachidonic acid (AA) from cellular phospholipid reservoirs. AA is metabolized to  
20 a number of potent inflammatory mediators in a process referred to as the arachidonic acid cascade.

Several acute and chronic clinical conditions have been characterized by elevated serum or local PLA<sub>2</sub> activity (see Table 1, below).

Table 1. Clinical Conditions Associated with PLA<sub>2</sub> Activity

	<u>Diseases</u>	<u>Sites</u>
5	Rheumatoid arthritis	Serum, synovial fluid, WBC
	Collagen vascular diseases	Serum
	Pancreatitis	Serum
	Peritonitis	Peritoneal fluid and cells
	Septic shock	Serum
10	ARDS*	Serum and alveolar fluid
	Acute renal failure	Serum
	Autoimmune uveitis	Serum, aqueous humor
	Bronchial asthma	Bronchial fluid

\* Adult respiratory distress syndrome

15

There are no effective PLA<sub>2</sub> inhibitors presently available for clinical use. To date, only a few PLA<sub>2</sub> inhibitors have progressed into clinical trials, but none have qualified for commercial marketing.

Fibronectin (Fn) is a 200 kDa glycoprotein which exists in several different forms and is secreted by different tissues. Fn is an essential protein and targeted disruption of the Fn gene in mice showed that it has a central role in embryogenesis. Fn also plays a key role in inflammation, cell adhesion, tissue repair and fibrosis, and is deposited at the site of injury. Plasma fibronectin (pFn) is secreted by the liver and circulates in the plasma. In the lung, cellular Fn (cFn) is secreted upon inflammation and injury. Both types of Fn are chemotactic factors for inflammatory cells and fibroblasts. Large numbers of inflammatory cells and fibroblasts infiltrate the lung during inflammatory episodes, which can lead to pulmonary fibrosis and ultimately death. Elevated levels of Fn have been detected in human clinical conditions such as neonatal RDS and BPD of the lung, and glomerular nephropathy of the kidney.

### The Role of UG

Amino acid analysis of purified human UG reveals that it is structurally similar but not identical to other "UG-like" proteins, e.g. rabbit UG. 39 of 70 amino acids are identical between human and rabbit UG (see Figure 1). The "UG-like" proteins, including human UG/CC10, rat CC10, mouse CC10, and rabbit UG, exhibit species-specific and tissue-specific antigenic differences, as well as differences in their tissue distribution and biochemical activities *in vitro*. UG-like proteins have been described in many different contexts with regard to tissue and species of origin, including rat lung, human urine, sputum, blood components, rabbit uterus, rat and human prostate, and human lung. At present there are no known physiological roles for these proteins.

Despite years of study, the biological roles of these proteins *in vivo* remain unclear. The absence of structural identity among UG-like proteins makes it impossible to predict whether a protein will possess *in vivo* therapeutic function in humans based on *in vitro* or other activity exhibited by a structurally related protein. For example, human uteroglobin binds less than 5% of the amount of progesterone as rabbit UG binds in the same assay. Human UG has a lower isoelectric point (4.6) than rabbit UG (5.4).

Stripp *et al.* (1996) have reported studies on a uteroglobin knockout mouse generated to eliminate expression of uteroglobin. The mouse has Clara cells which exhibit odd intracellular structures in place of uteroglobin secretion granules, but there is no other phenotype. This observation is highly significant because pulmonary function accompanied by pulmonary inflammation and fibrosis was expected. Moreover, this knockout mouse showed no evidence of renal, pancreatic, or reproductive abnormality, indicating that the uteroglobin protein had no significant role in controlling inflammation or fibrosis *in vivo*.

## OBJECTS OF THE INVENTION

Therefore, it is an object of the present invention to provide a pharmaceutical composition including a PLA<sub>2</sub> inhibiting effective amount of recombinant human uteroglobin (rhUG) or a fragment or derivative thereof and a pharmaceutically acceptable carrier or diluent.

It is a further object of the invention to provide a pharmaceutical composition including a fibronectin binding effective amount of recombinant human uteroglobin or a fragment or derivative thereof and a pharmaceutically acceptable carrier or diluent.

It is an additional object of the present invention to provide a pharmaceutical composition including a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of an active agent for treatment of a target indication.

Further, it is an object of the invention to provide a method for inhibiting PLA<sub>2</sub> enzymes *in vivo* in a mammal in need of such treatment, wherein the method includes administering to a mammal a PLA<sub>2</sub> inhibiting effective amount of native or recombinant human uteroglobin or a fragment or derivative thereof.

Still further, it is an object of the present invention to provide a method for treating or preventing an inflammatory condition in a patient in need of such treatment, wherein the method includes administering an anti-inflammatory effective amount of native or recombinant UG or a fragment or derivative thereof.

It is an object of the present invention to provide a method for treating or preventing a fibrotic condition in a patient in need of such treatment, wherein the method includes administering a fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

It is an additional object of the present invention to provide a method for treating or preventing an inflammatory or fibrotic condition characterized by a deficiency of endogenous UG, wherein the method includes administering a compensating amount of native or recombinant UG or a fragment or derivative thereof.

It is a further object of the present invention to provide a cosmetic composition comprising a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

5 Additionally, it is an object of the present invention to provide a blood supplement comprising a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

Finally, it is an object of the present invention to provide an assay quantitating uteroglobin-fibronectin complexes in a clinical sample.

10

### SUMMARY OF THE INVENTION

It has now been found that uteroglobin plays a central physiological role in inhibition of PLA<sub>2</sub>s and in prevention of fibronectin deposition and fibrosis *in vivo*. A combination of experiments performed in a new strain of transgenic uteroglobin "knockout" mice, and in a monkey model of neonatal respiratory distress syndrome (RDS) which involves pulmonary inflammation and fibrosis demonstrate these effects. 15 The uteroglobin knockout mice of the present invention (hereinafter the "UG KO mice/mouse") exhibit lethal glomerular nephropathy and renal parenchymal fibrosis, as early and late onset diseases, respectively. Administration of exogenous Fn to normal mice causes Fn deposition in the kidneys, but administration of equimolar 20 amounts of Fn and rhUG does not.

Reduction of PLA<sub>2</sub> activity *in vivo* has been demonstrated in the presence of rhUG. In a first experiment, the phenotype of the UG KO mice revealed that serum PLA<sub>2</sub> activity is significantly elevated in the absence of UG, compared to PLA<sub>2</sub> activity in littermates possessing a functional UG gene. In a second experiment, 25 administration of rhUG to pre-term monkeys suffering from RDS was shown to inhibit PLA<sub>2</sub> activity in the extracellular fluids of the lungs.

Other experiments demonstrate that *in vitro* PLA<sub>2</sub> can degrade the artificial surfactant (typically Survanta) used in treatment of RDS and that UG can inhibit this degradation. These experiments demonstrate that UG mediates PLA<sub>2</sub> inhibition and 30 Fn deposition *in vivo* following intratracheal or intravenous administration.



Experiments with the uteroglobin knockout mouse demonstrate that rhUG may be used to treat conditions in which uteroglobin is found to be deficient or the protein itself bears a loss-of-function mutation. It has now been discovered that rhUG may be used to treat or prevent inflammatory or fibrotic conditions in which functional  
5 endogenous uteroglobin is deficient in the circulation or at the site of inflammation or fibrosis. Reductions in the levels of hUG in serum and/or broncho-alveolar lavage fluids have been found in certain pulmonary inflammatory or fibrotic conditions, including pre-term infants at risk for developing neonatal BPD. It has been found that UG may be used to supplement deficient or defective endogenous uteroglobin to  
10 prevent or treat such inflammatory and fibrotic conditions.

According to one aspect, the invention provides a method of treating an inflammatory condition *in vivo* comprising administering to a patient in need of such treatment an anti-inflammatory effective amount of UG.

According to a further aspect, the invention provides a method of inhibiting  
15 soluble PLA<sub>2</sub> enzymes *in vivo*, which comprises administering to a patient in need of such treatment a PLA<sub>2</sub> inhibiting effective amount of UG.

In accordance with yet a further aspect, the invention provides a method for treating or preventing a fibrotic condition, which comprises administering to a patient in need of such treatment a fibronectin binding effective amount of UG.

20 A further aspect of the invention provides a method of treating or preventing fibrillogenesis by adding a fibronectin binding amount of UG.

In accordance with a further aspect, the invention provides a method for treating or preventing an inflammatory or fibrotic condition characterized by a deficiency of endogenous functional UG, which comprises administering to a patient  
25 in need of such treatment a compensating amount of UG.

The invention also provides pharmaceutical compositions comprising an effective amount of rhUG in association with a pharmaceutically acceptable carrier or diluent. The compositions may take the form of injectable solutions, and liquids or semi-aerosols for intratracheal administration.

According to a further aspect, the invention provides pharmaceutical compositions comprising UG and a lung surfactant (e.g. Survanta (a bovine lung extract from Abbott Labs) and Exosurf (a chemically synthetic lung surfactant from Glaxo-Wellcome)) in association with a pharmaceutically acceptable carrier or diluent.

5       The invention also includes pharmaceutical compositions containing a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of rhUG, an active agent for treatment of a target indication and a carrier. The PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of rhUG reduces inflammation and thereby insures that an effective amount of the active agent reaches the treatment site.

10       In another aspect, the invention provides an assay for quantitating uteroglobin-fibronectin complexes in a clinical sample, wherein a clinical sample suspected of containing a uteroglobin-fibronectin complex is contacted with an antigen capture agent, for example a monospecific rabbit polyclonal antibody, immobilized on an insoluble support; an antigen detection agent, for example an antibody specific for  
15   fibronectin, is added to the sample; and the presence of any complex bound to the support is detected using, for example, a secondary antibody, e.g., anti-IgG antibody conjugated to an enzyme such as horse radish peroxidase using a standard enzymatic reaction wherein the enzyme substrate is converted to a chromogenic or fluorogenic compound which is quantitated using standard spectrophotometric or fluorometric  
20   apparatus.

The invention also provides a cosmetic composition and a blood supplement including a PLA<sub>2</sub> inhibiting and/or fibronectin binding effective amount of human uteroglobin and a pharmaceutically acceptable carrier or diluent.

25

#### BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in more detail, with reference to the accompanying drawings, in which:

FIGURE 1 shows an alignment of UG-like proteins;

FIGURE 2A shows the intended targeting construct of the transgenic UG knockout mouse; the restriction sites are B - BamIII, E = EcoRI, H = HindIII; FIGURES 2B-2D show verification of the genetic construct in progeny of transgenic embryos by PCR and Southern blot analyses; (B) southern blot analyses of the targeted ES R1 cell clones, wherein Wt = wild type; (C) representative PCR analyses of genomic DNA from tail biopsies of offspring; the genotypes and their corresponding PCR products are as follows: UG<sup>+/+</sup>, 304 bp; UG<sup>+/-</sup>, 304 and 667 bp; UG<sup>-/-</sup>, 667 bp; (D) southern blot of mouse tail genomic DNA; FIGURE 2E shows confirmation of the absence of UG-mRNA in the lung tissues of UG<sup>-/-</sup> mice by RT-PCR analysis; RT-PCR analyses of total RNA extracted from the lung tissues of littermates with UG<sup>+/+</sup>, UG<sup>+/-</sup>, and UG<sup>-/-</sup> genotypes; a 273 bp RT-PCR product was detectable in the lungs of UG<sup>+/+</sup> and UG<sup>+/-</sup> but lacking from those of UG<sup>-/-</sup> mice; FIGURE 2F shows confirmation of the absence of UG protein in the lungs of UG<sup>-/-</sup> mice by Western analysis; proteins (30 micrograms each) from lung lysate were resolved by electrophoresis using 4-20% gradient SDS-Page under non-reducing conditions and immunoblotted using rabbit anti-mouse UG; FIGURE 2G shows confirmation of the absence of UG in lung tissue sections of the UG<sup>-/-</sup> mice using immunohistochemical methods in bronchiolar epithelial cells; the dark staining over the bronchiolar epithelial cells of UG<sup>+/+</sup> mouse (upper panel) indicates UG immunoreactivity; note the absence of immunoreactivity in UG<sup>-/-</sup> mouse lungs (lower panel).

FIGURES 3A-3J compare histopathological analyses of kidney sections from normal versus UG<sup>-/-</sup> mice, showing abnormal parenchymal fibrosis and glomerular Fn deposition in the knockout mice only; H & E staining of kidney sections from a UG<sup>+/+</sup> (A) and its UG<sup>-/-</sup> (B) littermate; (C) kidney section of a 10 month old mouse with severe parenchymal fibrosis; (D) a region of the same mouse kidney in (C) showing renal tubular hyperplasia (magnification 40X, g = glomerulus; f = fibrosis; t = tubule); (E) transmission electron microscopy of the glomerular deposit of a UG<sup>-/-</sup> mouse with severe renal disease (magnification 6000X); (F) the inset in (E) is magnified 60,000X, which shows the long striated fibrillar structures indicative of

collagen (col) and short diffuse ones consistent with Fn fibrils; (G) Fn immunofluorescence of a kidney section from a UG<sup>+/+</sup> mouse using murine Fn-antibody; (H) Fn-immunofluorescence of a kidney section from a UG<sup>-/-</sup> mouse with severe renal disease; Mason's trichrome staining of the kidney sections with UG<sup>+/+</sup> (I) and UG<sup>-/-</sup> (J) mice; the bluish staining over the glomeruli of UG<sup>-/-</sup> mouse kidney section is collagen (magnification approximately 40X).

FIGURE 4A shows the presence of Fn aggregates only in the kidneys of the UG<sup>-/-</sup> mice; immunoprecipitation and western blotting of Fn from plasma, kidney, and liver of UG<sup>+/+</sup> and UG<sup>-/-</sup> mice; a multimeric Fn band (bold arrow) was detected only in the kidney lysates of UG<sup>-/-</sup> mice.

FIGURES 4B and 4C show the formation of UG-Fn complexes *in vitro*; (B) equimolar concentrations of UG and Fn were incubated, immunoprecipitated with and detected by Western blotting with either Fn or UG antibody; the immunoprecipitates contain both Fn (lane 2, upper panel) and UG (lane 2, lower panel); lanes 1 of both panels represent Fn and UG standards; (C) equimolar concentrations of <sup>125</sup>I-UG and Fn were incubated at 4°C for 1 hour and the resulting complex was resolved by electrophoresis on 6% non-reducing, non-denaturing polyacrylamide gels; lane 1, coomassie blue stained Fn-UG heteromer; lane 2, its autoradiogram.

FIGURE 4D shows the presence of UG-Fn complexes in the plasma of normal but not UG<sup>-/-</sup> mice; immunoprecipitation of plasma from UG<sup>+/+</sup> and UG<sup>-/-</sup> mice with Fn-antibody and western blotting with Fn and UG antibodies; Fn (upper panel); UG (lower panel); std = standards for UG and Fn.

FIGURE 4E shows the dose-dependent inhibition of Fn self-aggregation by UG *in vitro*; affinity-crosslinking of <sup>125</sup>I-Fn with unlabeled Fn in the absence (lane 2) and presence of varying amounts of UG (lanes 3-5); the intensity of the very high molecular weight, radioactive Fn band (lane 2) formed in the absence of UG is reduced in a dose-dependent manner; lane 1, <sup>125</sup>I-Fn with unlabeled Fn in the absence of UG and DSS; open arrowhead - multimeric Fn; lower thin arrow = 220 kDa Fn.

FIGURE 4F shows the inhibition of Fn-collagen complex formation by UG; affinity crosslinking of  $^{125}\text{I}$ -collagen I with unlabeled Fn in the absence of (lane 3) and presence (lane 4) of UG; lane 1, coomassie blue-stained collagen I;  $\alpha_1$ - $\alpha_1$  chain of collagen I and  $\alpha_2$ - $\alpha_2$  chain of collagen I; lane 2,  $^{125}\text{I}$ -collagen I and unlabeled Fn in the absence of UG and DSS.

FIGURES 5A-5F show the immunohistochemical analysis of Fn deposition in the kidneys of normal and  $\text{UG}^{-/-}$  mice only in the absence of UG; (A) kidney section of a wild-type mouse that received a mixture of equimolar concentrations of Fn and UG intravenously; (B)  $\text{UG}^{+/+}$  mouse that received the same dose of Fn as in (A) but without UG; (C) apparently healthy,  $\text{UG}^{-/-}$  mouse receiving a mixture of Fn and UG; (D)  $\text{UG}^{-/-}$  mouse receiving Fn alone (same dose as in (C)), but without UG; (E) Fn-fibrillogenesis by cultured cells grown in medium supplemented with soluble hFn alone; (F) a cell culture identical to one in (E) which was fed with medium containing a mixture of equimolar concentrations of soluble hFn and UG (magnification 40X, g = glomerulus).

FIGURES 6A-6B show the format for a diagnostic assay to detect UG-Fn complexes in clinical samples.

FIGURE 7 shows the passage of UG dimer through an 8.0 kDa MWCO dialysis membrane but not a 3.5 kDa MWCO dialysis membrane.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

### rhUG

The rhUG of the invention has substantially the same amino acid sequence as that of the native human UG protein. An amino acid sequence having "substantially the same" amino acid sequence as that of the native human protein includes rhUG having at least 75% identity to the native human protein. In a preferred embodiment, rhUG has at least 85% identity, and in a most preferred embodiment, rhUG has at least 98% identity to the native UG.

Also included in the method of the present invention is the use of fragments or derivatives of UG. A "fragment" of UG refers to a portion of the native hUG

amino acid sequence having six or more contiguous amino acids of the native protein sequence. The term "derivative" refers to peptide analogs of UG, including one or more amino acid substitutions and/or the addition of one or more chemical moieties, e.g., acylating agents or sulfonating agents, with the proviso that the derivative  
 5 retains the biological activity of the parent molecule.

Further, the UG used in the method of the present invention is substantially pure. The term "substantially pure" refers to UG having a purity of about 75% to about 100%. In a preferred embodiment, UG has a purity of about 90% to about 100%, and in the most preferred embodiment, UG has a purity of at least 95%.

#### 10 Clinical Uses of UG

The invention provides, in another aspect, a method of treating or preventing an inflammatory or fibrotic condition comprising administering to a mammal, which may be animal or human, an effective amount of UG.

The following non-limiting list of conditions are representative examples of  
 15 those associated with UG deficiencies, excessive PLA<sub>2</sub> activity, and fibronectin deposition.

**Table 2. Clinical Uses of Recombinant Uteroglobin  
(Grouped by UG Property)**

20	<u>UG Property</u>	<u>Condition(s)</u>
	UG deficiency	(1) Neonatal broncho-pulmonary dysplasia; (2) Complications of hemodialysis; (3) Bleomycin lung; (4) Chronic obstructive pulmonary disease; (5) Emphysema
	Excessive PLA <sub>2</sub> activity (inflammation)	(1) Systemic inflammation; (2) Asthma; (3) Cystic fibrosis; (4) Ocular inflammation, including Autoimmune uveitis and corneal transplant surgery; (5) Conditions associated with obstetrics and gynecology, including premature labor and fertility (ex vivo)
25	Excessive PLA <sub>2</sub> activity (Immune modulation)	(1) Autoimmune diseases, including rheumatoid arthritis, Type I diabetes, Inflammatory bowel disease, and Crohn's disease; (2) Transplanted organ rejection
	Fibronectin deposition	(1) Renal fibroses (2) Pulmonary fibroses, including idiopathic pulmonary fibrosis; (3) Vascular fibrosis

Typical relationships between UG deficiency, PLA<sub>2</sub> activity and fibronectin aggregation and deposition in human inflammatory/fibrotic conditions are summarized below.

5

#### **Neonatal Broncho-Pulmonary Dysplasia (Neonatal BPD)**

Neonatal BPD is characterized by severe inflammation and irreversible fibrosis of lung tissue in newborn infants, usually as a result of respiratory distress syndrome (RDS). However, this condition may also be caused by meconium aspiration syndrome or infection.

10

A deficiency of hUG has been implicated in this condition because the synthesis of pulmonary hUG may be coregulated with surfactant, which starts late in gestation. Thus, severely premature neonates may lack UG as well as surfactant. hUG deficiency may cause increased PLA<sub>2</sub> activity and Fn-related fibrosis, which are associated with the inflammation and fibrosis seen in neonatal BPD. Some infants do not respond to synthetic surfactant, which may be due to excess PLA<sub>2</sub> activity. Thus, UG may be used to treat neonatal BPD.

15

The preferred route of administration is direct instillation via the endotracheal or the systemic routes.

20

#### **Multiple Organ Failure (MOF)**

Excessive PLA<sub>2</sub> activity has been implicated in MOF due to bacterial sepsis or trauma. This condition is characterized by a systemic inflammatory response, involving rapid, massive tissue damage and loss of organ function in the lungs, kidney, pancreas, intestines, and vasculature. Recent evidence points to the MOF trigger as elevated systemic soluble phospholipase A<sub>2</sub> activity, its direct lysis of tissue cell membranes, and hydrolysis of essential phospholipids, such as lung surfactants. Attempts to inhibit PLA<sub>2</sub> directly in clinical settings have been unsuccessful.

25

In MOF, the amount of endogenous UG is insufficient to counter the super-activation of PLA<sub>2</sub>. Exogenously supplied UG can be used to combat MOF.

30

Remote organ failure (ROF) involves damage to organs other than the organ primarily affected by trauma or infection. Often remote organ failure involves more than one remote organ, resulting in multiple organ failure. For example, pancreatitis is an inflammation of the pancreas in response to alcohol intake, infection, or trauma, that may result in adult respiratory distress syndrome (ARDS), acute renal failure (ARF), and systemic shock. An episode of inflammatory bowel disease or peritonitis can result in ROF/MOF. ROF/MOF is associated with high levels of circulating, activated PLA<sub>2</sub>. The systemic application of hUG could prevent ROF/MOF. The immediate injection of UG in patients with ROF/MOF, could reduce the severity or eliminate the PLA<sub>2</sub> mediated organ failure and shock.

### Pancreatitis

All forms of pancreatitis involve elevated Type I soluble PLA<sub>2</sub> activity, both systemic and local. Pancreatitis often results in pulmonary insufficiency or ARDS, characterized by elevated soluble PLA<sub>2</sub> activity in the lungs. Therefore, as an inhibitor of soluble Type I PLA<sub>2</sub>s *in vivo*, UG is an excellent candidate for treatment of two forms of acute pancreatitis, and as a preventative measure of pulmonary insufficiency in all acute forms of pancreatitis.

The preferred route of administration is by the intravenous route.

20

### Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD), including ulcerative colitis, diverticulitis, and Crohn's disease, is characterized by elevated local production and activity of Type II soluble PLA<sub>2</sub>. Circulating soluble PLA<sub>2</sub> activity may also be elevated in IBD. IBD causes pulmonary insufficiency or ARDS in severe cases, as a result of elevated PLA<sub>2</sub> activity (which is similar to pancreatitis).

The rationale for the application of exogenous UG in IBD is identical to that of pancreatitis: to downregulate the inflammatory response by inhibiting PLA<sub>2</sub>, Fc aggregation and/or deposition and to prevent remote organ involvement (lungs and kidneys).

30



The preferred route of administration is by the intravenous route in hospitalized patients.

### **Bacterial Pneumonia**

- 5           BAL fluids of patients who have survived bacterial pneumonia were shown to have 2-3X higher levels of UG than those who died. Bacterial infection of the lungs may overactivate the endogenous soluble PLA<sub>2</sub>. UG may be administered to inhibit or control this effect.

10           The preferred route of administration is via the intratracheal route if the patient is intubated or intravenous if not.

### **Complications of Dialysis**

- 15           The major complication of dialysis is thromboses, i.e., spontaneously formed blood clots. These often plug the vascular access port, impairing treatment, as well as causing ischemic, sometimes life-threatening episodes, in the patient. A second problem with hemodialysis patients is inflammation and/or fibrosis of the proximal vein which returns the dialyzed blood to the patient's main circulation. Fibrosis of the proximal vein is usually detected as an increase in resistance, or pressure, against the return of the dialyzed blood. A third problem is fibrosis and closure of the vascular access site, or fistula. A fourth problem is accelerated atherosclerosis and a fifth is loss of residual renal function, most likely due to Fn deposition.

- 25           The possibility that endogenous UG is dialyzed away during the procedure provides an explanation for these problems. The selective removal of endogenous UG leaves circulating Fn free to aggregate, forming the foci for blood clot formation or to deposit on red blood cells, priming them for a clotting response by sticking to each other or to the vascular lumen. Transglutaminases (TGs) are enzymes responsible for building macromolecular lattices found in basement membranes, skin and blood clots. In the absence of free UG competing as a substrate for activated TGs, Fn and other components of blood clots are crosslinked.

Inflammation and fibrosis of both the proximal vein and the vascular access site, as well as accelerated atherosclerosis, may be explained by the deposition of Fn in the vascular lumen. Fibronectin deposition on the vascular endothelia promotes platelet and white blood cell adherence, both of which may be aggravated in the absence of PLA<sub>2</sub> inhibition. Vascular deposits of Fn may also promote local deposits of fat, cholesterol and protein found in atherosclerotic plaque. Fibronectin is known to be a major component of atherosclerotic plaque, as well as renal glomerular deposits associated with nephropathy and loss of primary and residual renal function. Therefore, UG administration may reduce or eliminate these problems by reducing inflammation and fibronectin deposition.

The preferred route of administration of UG would be intravenous infusion before, during or after dialysis.

Alternatively, the loss of endogenous UG may be prevented by addition of UG to the dialysis buffer or precoating the dialysis membrane with UG or both.

15

### Organ Transplants

The term "organ" refers, for example, to solid organs, such as kidney, liver and heart, as well as bone marrow, cornea and skin.

There are two types of organ transplant rejection: acute and chronic. Acute rejection is an inflammatory process involving PLA<sub>2</sub> activity and infiltration by inflammatory cells that often destroys the graft.

Chronic rejection involves Fn-mediated fibrosis of the graft, including atherosclerosis confined to the graft. Thus, administration of UG may be used to treat or prevent both acute and chronic graft rejection.

25 The preferred route of administration is by injection.

Another aspect of organ transplantation is ischemia of the organ before removal from the donor, during transport and in the recipient, which contributes to acute rejection. Ischemia is known to result in elevated PLA<sub>2</sub> activity and tissue necrosis. Hence, UG could be used to prevent such ischemia. The preferred form

of UG is as a perfusion liquid or storage buffer in which the ex vivo organ is preserved.

#### **Prevention of Type I Diabetes**

5

Type 1 diabetes arises from the destruction of pancreatic tissue by an autoimmune response. The pancreas normally secretes soluble PLA<sub>2</sub>s and hUG into the circulation. Necrotic lesions have been reported in the pancreas of the

10 "UG KO mouse").

In the absence of uteroglobin, the UG KO mouse exhibits similar pancreatic tissue destruction which could trigger an autoimmune response. Thus UG may be used to prevent or halt the slow progression of Type 1 diabetes. The preferred route of administration is by injection.

15

#### **Prevention and Treatment of Nephropathy**

Renal Fn deposits and fibrosis in the UG KO mouse are similar to Fn deposits and fibrosis in human nephropathies. Thus, UG administration may prevent or slow the progression of nephropathy in patients at risk, such as Type II diabetes.

20

#### **Prevention and Treatment of Ocular Inflammation**

Ocular inflammation, including uveitis, retinitis, and inflammation following surgery, is characterized by increased PLA<sub>2</sub> activity. Therefore, UG may be administered topically, intraocularly, or systemically to reduce ocular inflammation.

25

#### **Arteriosclerosis**

Arteriosclerosis is a fibrotic thickening of blood vessels throughout the body. It is initiated and/or mediated by Fn deposition on the walls of the vasculature. Atherosclerosis is a form of arteriosclerosis involving cholesterol deposition, in addition to Fn deposition. Therefore, UG may be administered to prevent or reduce arteriosclerosis.

30

**Acute Renal Failure**

Acute renal failure (ARF) is typically a consequence of remote organ inflammation, infection or direct trauma, which results in release and activation of soluble PLA<sub>2</sub> in the circulation. Damage to the kidneys during ARF can be quite  
 5 severe, with acute tissue damage promoted by inflammation and may resolve into fibrosis of the kidney, leading to reduced kidney function in the long term. The anti-inflammatory and anti-fibrotic properties of UG are particularly relevant in the kidney as shown by the UG KO mouse.

The preferred route of administration is by injection or systemic  
 10 administration.

Overall, the following non-limiting list of conditions are those associated with inhibition of PLA<sub>2</sub> and/or fibronectin deposition, each of which are candidates for treatment or prevention by the method of present invention:

15

- |                           |   |
|---------------------------|---|
| <b>Joint/Bone:</b>        | rheumatoid arthritis;   |
| <b>Autoimmune:</b>        | rheumatoid arthritis, multiple sclerosis, Type 1 diabetes, uveitis, psoriasis, systemic lupus erythematosus (SLE), and Crohn's disease;   |
| 20 <b>Pancreas:</b>       | pancreatitis;   |
| <b>Peritoneum:</b>        | peritonitis, appendicitis;  |
| <b>Vascular/systemic:</b> | septic shock; collagen vascular disease, arteriosclerosis, atherosclerosis, anaphylactic <del>shock</del> , schistosomiasis, and trauma-induced shock;  |
| 25 <b>Renal:</b>          | acute renal failure, bacterial infection of the kidneys, inflammation due to renal tumors, prevention of fibrosis resulting from chemotherapy or antibody therapy, prevention of diabetic nephropathy, prevention and/or treatment of idiopathic nephropathy; |
| 30 <b>Liver:</b>          | hepatitis, viral hepatitis, and cirrhosis;  |

	<b>Bladder:</b>	cystitis, inflammation of the urethra, inflammation of the ureter, and bladder inflammation such as interstitial cystitis;
5	<b>Reproductive/female:</b>	vaginitis, inflamed cervix, pelvic inflammatory disease, inflammation of the ovary (salpingitis), endometriosis, vaginal candidiasis, and inflammation or fibrosis of the fallopian tubes;
10	<b>Reproductive/male:</b>	penile inflammation, prostate inflammation, inflammation of seminal tubules and vesicles, testicular inflammation, and inflammation of vas deferens, epididymis, and prostate gland;
15	<b>Ocular:</b>	uveitis, retinitis, trauma, burn damage due to chemical or smoke, ocular inflammation due to CMV retinitis, conjunctivitis (bacterial infection), viral infection, ocular inflammation due to infectious agent, ocular inflammation following ocular surgery, including cataract removal, laser surgery, corneal transplant, tumor removal, ocular inflammation due to retinoblastoma (tumor), ocular inflammation due to radiation exposure, inflammation due to allergic response;
20	<b>Heart:</b>	Endocarditis
25	<b>Lungs:</b>	bronchial asthma, ARDS, pneumonia, idiopathic pulmonary fibrosis, pulmonary fibrosis resulting from chemotherapy (bleomycin, methotrexate), pulmonary fibrosis resulting from exposure to environmental chemicals (asbestos, cleaning fluids, pollutants, e.g. dioxin and PCB's in automobile exhaust), smoke inhalation, pulmonary inflammation during recovery from drowning, and neonatal RDS;
30		

- 5                   **Gut:**                   inflammatory bowel disease, colitis, Crohn's disease, diverticulitis, neonatal necrotizing enterocolitis, inflammation due to an infectious agent, rotavirus, polio virus, HIV, stomach ulcers, gastro-esophageal reflux disease, and tonsillitis;
- Hemorrhoids;**
- 10                   **Transplants:**               administration following transplant surgery for any organ or tissue to control inflammation or fibrosis and rejections;
- Ears:**                   Otitis media; and
- Skin:**                   psoriasis, hives, allergic and dermatitis, scleroderma, contact dermatitis, chemical dermatitis (due to poison ivy, poison oak, and exposure to chemicals like PCB's, chlorine, ammonia, (cleaning agents, toxic agents).
- 15

Moreover, UG may be administered either alone or in combination with other active agents or compositions typically used in the treatment or prevention of the above-identified disease conditions. Such active agents or compositions include, but

20 are not limited to steroids, non-steroidal anti-inflammatories (NSAIDs), chemotherapeutics, analgesics, immunotherapeutics, antiviral agents, antifungal agents, vaccines, immunosuppressants, hematopoietic growth factors, hormones, cytokines, antibodies, antithrombotics, cardiovascular drugs, or fertility drugs. Also included are oral tolerance drugs, vitamins and minerals.

25               Moreover, UG may be administered as a carrier for the suppression of inflammation at the site of injection or administration of another therapeutic or prophylactic agent to suppress inflammation or an immune response caused by such agent. In this regard, UG would allow for administration of an effective amount of the active agent to the target indication.

UG may also be administered as a cosmetic composition for use in limiting fibrosis, scar or keloid formation resulting from wound healing, or during a dermal inflammatory response.

Further, UG may be used as a blood supplement, i.e., as an additive to  
5 synthetic or donated human blood.

Finally, UG may be used to enhance the rate of artificial insemination by adding to sperm, in vitro fertilized eggs, and embryos prior to transfer to the maternal uterus in humans and other mammals.

The present invention relates to the use of UG in the prevention or treatment  
10 of PLA<sub>2</sub> and fibronectin associated conditions. With regard to prevention of a disease condition, "prevention" refers to preventing the development of disease in a susceptible or potentially susceptible population, or limiting its severity or progression, whereas the term "treatment" refers to the amelioration of a disease or pathological condition.

15 UG may be administered intravenously or, in the case of treatment of neonatal RDS/BPD and adult RDS, in the form of a liquid or semi-aerosol via the intratracheal tube. Other viable routes of administration include topical, ocular, dermal, transdermal, anal, systemic, intramuscular, slow release, oral, vaginal, intraduodenal, intraperitoneal, and intracolonic. Such compositions can be administered to a subject  
20 or patient in need of such administration in dosages and by techniques well known to those skilled in the medical, nutritional or veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular subject or patient, and the route of administration. The compositions of the present invention may also be administered in a controlled-release formulation. The compositions can be co-  
25 administered or sequentially administered with other active agents, again, taking into consideration such factors as the age, sex, weight, and condition of the particular subject or patient, and, the route of administration.

Examples of compositions of the invention include edible compositions for oral administration such as solid or liquid formulations, for instance, capsules, tablets,  
30 pills, and the like liquid preparations for orifice, e.g., oral, nasal, anal, vaginal etc.,

formulation such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration), such as sterile suspensions or emulsions. However, the active ingredient in the compositions may complex with proteins such that when  
5 administered into the bloodstream, clotting may occur due to precipitation of blood proteins; and, the skilled artisan should take this into account.

In such compositions UG may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, DMSO, ethanol, or the like. UG can be provided in lyophilized form for reconstituting, for instance, in  
10 isotonic aqueous, saline, glucose, or DMSO buffer. In certain saline solutions, some precipitation of rhUG has been observed; and this observation may be employed as a means to isolate inventive compounds, e.g., by a "salting out" procedure.

Further, the invention also comprehends a kit wherein UG is provided. The kit can include a separate container containing a suitable carrier, diluent or excipient.  
15 The kit can include an additional agent which reduces or alleviates the ill effects of the above-identified conditions for co- or sequential-administration. The additional agent(s) can be provided in separate container(s) or in admixture with UG. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration.

20 The invention also contemplates a method for treating or preventing an inflammatory or fibrotic condition characterized by a deficiency of endogenous functional UG, which comprises administering to a patient in need of such treatment a compensating amount of UG. The term "compensating amount" means an amount of UG required to bring the local pulmonary or systemic concentration of total UG  
25 (endogenous functional UG and exogenous UG) to within its normal range. More specifically, the normal range for local pulmonary concentration of endogenous UG is about >50 micrograms UG/milligram albumin or >50 micrograms/liter. The normal range for serum UG concentration is >15 micrograms/liter.

The compositions of the invention comprise native and/or recombinant hUG  
30 in an amount effective to achieve the intended purpose, namely increased plasma or



tissue levels of UG to produce the desired effect of selective inhibition of PLA<sub>2</sub>s and reduced inflammation and/or binding of fibronectin to mitigate its aggregation and/or deposition in fibrotic conditions. The compositions comprise an effective amount of substantially pure native and/or recombinant human UG, in association with a  
5 pharmaceutically acceptable carrier or diluent.

In a further aspect, the compositions of the invention comprise an effective amount of native and/or recombinant hUG and lung surfactant, in association with a pharmaceutically acceptable carrier or diluent. Local intratracheal administration of UG to the lungs, sufficient for inhibition of PLA<sub>2</sub> activity and/or fibronectin  
10 deposition requires quantities of substantially pure UG in the range of 0.2 µg/kg to 500 mg/kg of protein in single or multiple dosages. The UG is usually administered in an amount of a single bolus of 20 ng/kg to 500 mg/kg, in single or multiple doses, or as a continuous infusion of up to 10 grams.

Native and/or recombinant hUG may also be administered in conjunction with  
15 artificial lung surfactant, such as Survanta, via the intratracheal route. UG and Survanta (5 mls/kg) are co-administered and UG does not bind surfactant, preventing it from functioning therapeutically. The lung surfactant is generally present in the composition an amount of about 10-90 % by weight, more usually about 20-80 % by weight. The surfactant, by virtue of its very low surface tension spreads out over the  
20 internal surface of the lungs, carrying the UG with it. Systemic administration of UG via intravenous injection, sufficient for inhibition of PLA<sub>2</sub> activity and/or fibronectin deposition, requires quantities of substantially pure native and/or recombinant hUG in the range of 0.5 µg to a continuous infusion of several grams of protein over an extended period of time (days).

25 Suitable formulations for injection and semi-aerosol intratracheal delivery include aqueous solutions of native and/or recombinant hUG optionally with viscosity modifiers and stabilizers.

The term "PLA<sub>2</sub> inhibiting effective amount" as used herein means the amount of UG which inhibits PLA<sub>2</sub> activity and which reduces or alleviates inflammation in  
30 the tissue or body of the patient. The term "fibronectin binding effective amount"

means that amount of UG which binds fibronectin to reduce aggregation and/or deposition thereof, and prevent or reduce fibrillogenesis or fibrosis. The term "anti-inflammatory amount" as used herein means the amount which reduces or alleviates inflammation in the tissue or body. Typically, the amount of UG administered to adults for the treatment of inflammatory and fibrotic conditions will be single boluses of 0.2  $\mu$ g/kg to 500 mg/kg or up to several grams administered over an extended period of time. For neonates, in the treatment of neonatal RDS, the range will typically be 50 nanograms/kg to 100 mg/kg in single boluses or up to 10 grams administered continuously over an extended period of time. Effective and safe rates of continuous infusion are between 50 ng/kg/hour to 500 mg/kg/hour.

### EXAMPLES

The invention will now be further described with reference to the following non-limiting examples. Parts and percentages are by weight unless otherwise stated.

#### Example 1: In Vivo Experiments

Recombinant human UG was obtained by the method of Mantile *et al.* (1993).

One male and one female of the species *P. cyanocephalus*, weighing approximately 400 grams each were delivered by C-section at 142 days of gestation. This is an established model of RDS (Coalson, J.J., et al. Baboon Model of BPD. II: Pathologic features. *Exp. Mol. Pathol.* 37: 355-350 (1982)).

After delivery, the infants were anesthetized with ketamine (10 mg/kg) and intubated with a 2.5 mm diameter endotracheal tube. Blood gases and pressure were monitored via an arterial line placed by percutaneous injection into the radial artery. A deep venous line was placed percutaneously into the saphenous vein through which fluids, antibiotics, and drugs were administered. Animals were maintained on servo-controlled infrared warmers and ventilated with a standard time-cycled, pressure-regulated ventilator with humidifiers maintained at 36-37°C. Initial setting were  $\text{FiO}_2$  1.0, rate 40/min., I/E ratio 1:1.5, positive end expiratory pressure (PHEP) at 4

cm H<sub>2</sub>O, and peak inspiratory pressure (PIP) as required for adequate chest excursion. FiO<sub>2</sub> was kept at 1.0 and PIP was regulated to maintain PaCO<sub>2</sub> at 40±10 torr. Blood gases, hematocrit, electrolytes, prothrombin time, partial thromboplastin time and dextrostix were monitored hourly. Blood drawn for studies was replaced  
5 volumetrically with heparinized adult baboon blood. Intravenous fluids were administered with electrolytes at 10 cc/kg/hr and were increased as needed when heart rate exceeded 180 beats/min. Sodium bicarbonate (2 meq/kg) was administered when the base deficit exceeded -10. Ampicillin (50 mg/kg/day in two divided doses) and Gentamicin (5 mg/kg/day in two divided doses) was given continuously for the  
10 duration of the experiment.

One animal received surfactant plus PBS (treatment no. 1), and the second animal (treatment no. 2) received surfactant plus two doses of 1 mg/kg of rhUG. Both surfactant and rhUG were administered directly to the lungs through the endotracheal tube. The surfactant used was Survanta (Ross Labs), a surfactant  
15 preparation derived from bovine lung tissue, containing surfactant apoproteins B and C in addition to phospholipids. The first dose of rhUG was given with the surfactant and the second administered four hours after the first. The animals were monitored for arterial blood gases, electrolytes and EKG. They were sacrificed 50 hours after the initiation of surfactant therapy. The lungs were lavaged at 24 and 48 hours with  
20 PBS containing protease inhibitors (PMSF, 10 µg/ml leupeptin, 10 µg/ml of pepstatin and bacitracin). They were frozen at -80°C until assayed for PLA<sub>2</sub> activity. Total proteins were determined by Bradford method (BioRad). The PLA<sub>2</sub> activity in the lung lavages were measured according to Levin et al. (1986; *supra*) and are presented in the following Table.

Table 3. Results of In Vivo Testing of UG

	Treatment #	Time	Lung lavage PLA <sub>2</sub> activity (ccpm/10 µg protein)
5	1	24 hr 48 hr	3030 2607
	2	24 hr 48 hr	1739 996

The data given above are the mean of two determinations. The results show that endotracheal administration of rhUG inhibits PLA<sub>2</sub> *in vivo*. The animals which received surfactant and rhUG had an appreciably lower PLA<sub>2</sub> activity in their lung lavage fluid compared with the animals that received surfactant without rhUG. The data confirm that administration of rhUG in conjunction with surfactant is beneficial in protecting surfactant phospholipids.

15

**Example 2: Inhibition of Hydrolysis of Artificial  
Surfactant by Soluble PLA<sub>2</sub> *in vitro***

RhUG inhibits hydrolysis of artificial surfactant by soluble PLA<sub>2</sub>s *in vitro*. Survanta is an artificial surfactant derived from bovine lung and is used to treat pre-term neonates with RDS and adults with RDS (ARDS). Hydrolysis of Survanta by a Group I soluble PLA<sub>2</sub>, i.e. porcine, pancreatic PLA<sub>2</sub> (Boehringer Mannheim) is characterized by its ability to compete as a substrate with a fluorescent phosphatidylcholine substrate (Cayman Chemicals), generating arachidonic acid as a product.

25

Survanta is a substrate for *in vitro* degradation by Group I soluble PLA<sub>2</sub>s. Survanta is rapidly degraded *in vitro* by PLA<sub>2</sub>s found in the extracellular fluids of a human lung. RhUG inhibits degradation of Survanta *in vitro*.

30

**Example 3: Construction of UG knockout mouse**

A transgenic UG KO mouse was created for the purpose of determining the role of uteroglobin in mammalian physiology, as well as to generate a model for UG as a therapeutic in several inflammatory clinical conditions. The first step was to

5 construct an appropriate DNA vector with which to target and interrupt the endogenous murine uteroglobin gene. The 3.2 kb BamHI-EcoRI DNA fragment containing exon 3 and flanking sequences of the uteroglobin gene from the 129/SVJ mouse strain (Ray, 1993) were subcloned into the corresponding sites of the pPNW vector as described in Lei et al (1996). A 0.9 kb fragment containing part of exon

10 2 and its upstream sequence was amplified by PCR (with primers Primer-L (from Intron 1): 5'-TTC CAA GGC AGA ACA TTT GAG AC-3'; Primer-R (from Exon 2): 5'-TCT GAG CCA GGG TTG AAA GG C-3') with NotI and XhoI restriction sites engineered into the termini for directional subcloning into the gene targeting vector. In this construct, 79 bp of Exon 2 encoding 27 amino acids were deleted.

15 The PCR fragment was placed upstream of the gene encoding neomycin resistance in pPNW, generating the gene targeting vector, pPNWUG. The vector is shown in Figure 2A, in which the PGK-neo cassette interrupts the uteroglobin gene, disrupting the protein coding sequence.

The pPNWUG gene targeting vector was linearized with NotI and

20 electroporated into ES R<sub>1</sub> cells according to Nagy, A., et al. PNAS 90:8424 (1993). Gancyclovir and G-418 selection of the electroporated cells yielded 156 clones. Southern (DNA) blot analysis identified a 5.1 kb HindIII fragment of the wild-type uteroglobin allele and an additional 8.2 kb HindIII fragment resulting from homologous recombination in three out of the 156 clones, shown in Figure 2B.

25 These ES R<sub>1</sub> clones were injected into C57BL/6 blastocysts according to Capecchi, Science 244: 1288 (1989). Two different lines of mice, descended from different chimeric founders, were generated. Heterozygous offspring (UG<sup>+/+</sup>) carrying the targeted uteroglobin gene locus were mated and the genotypes of the progeny were analyzed by PCR shown in Figure 2C, as well as Southern blot, shown in Figure 2D.

**Example 4: Verification of UG gene knockout and absence of murine UG (mUG) protein**

In order to verify that the homozygous knockout mice ( $UG^{-/-}$ ) did not possess any detectable mUG, the uteroglobin gene-targeted mice were tested for expression ofm UG-mRNA and mUG protein in several organs including the lungs. An experimental protocol was approved by the institutional animal care and use committee. Total RNAs were isolated from different organs of  $UG^{+/+}$ ,  $UG^{+/-}$ , and  $UG^{-/-}$  mice. The reverse transcribed-polymerase chain reaction (RT-PCR) was used to detect mUG-mRNA. Target molecules were reverse transcribed using a mUG-specific primer, mPr (5'- ATC TTG CTT ACA CAG AGG ACT TG-3'), and the cDNA generated was amplified using PCR primers mPr and mPl (5'-ATC GCC ATC ACA ATC ACT GT-3'). The PCR product was hybridized with an oligonucleotide probe, mPp (5'-ATC AGA GTC TGG TTA TGT GGC ATC C-3') derived from exon-2 of the UG gene sequence. The primers and the probe used in mouse GAPDH RT-PCR are as follows: mGAPDH-r (5'-GGC ATC GAA GGT GGA AGA GT-3'); mGAPDH-l (5'-ATG GCC TTC CGT GTT CCT AC-3'); mGAPDH-p (5'-GAA GGT GGT GAA GCA GGC ATC TGA GG-3'). Figure 2E shows that mUG-mRNA was detected in the lungs of  $UG^{+/+}$ , and  $UG^{+/-}$ , but not  $UG^{-/-}$  mice. Similar data (not shown) show that mUG-mRNA is not present in either the prostate or uteri of  $UG^{-/-}$  mice, but is present in the mice with an intact uteroglobin gene.

Immunoprecipitation and Western blot analyses of mUG protein in the lungs yielded similar corroborative results, shown in Figure 2F. Tissue lysates from the kidneys, liver, and the lungs of the  $UG^{+/+}$  and  $UG^{-/-}$  mice were prepared by homogenizing in a buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.2% deoxycholate, 150 mM NaCl, 5 mM EDTA) containing 2 mM phenylmethylsulfonyl fluoride and 20  $\mu$ g/mL each of aprotinin, leupeptin, and pepstatin A. The homogenates were centrifuged at 17,500 x g for 30 min at 4°C and immunoprecipitated as described (E. Harlow and D. Lane, Antibodies; a laboratory manual, 1st Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988) by incubating tissue lysates or plasma proteins (1 mg/mL) with rabbit antibody

against murine Fn (1:100 dilution). Co-immunoprecipitation of purified murine Fn (mFn) and rhUG (Mantile, G, et al., J. Biol. Chem. 267: 20343 (1993)) was performed by incubating equimolar concentrations of mFn with rhUG in the presence of 10% glycerol, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 4.3 mM sodium phosphate at 4°C for 1 hr., followed by adding anti-mFn antibody (1:100 dilution). Equal amounts of extracted tissue proteins (30 µg) or immunoprecipitates were resolved either on 4-20% or 6% SDS-polyacrylamide gels under reducing conditions, followed by Western blotting with rabbit antibodies against either murine Fn (1:2000 dilution) or UG (1:2000 dilution). No mUG was detected in tissues or fluids from the UG<sup>-/-</sup> mice, while tissues from UG<sup>+/+</sup> and UG<sup>+/-</sup> mice did contain the mUG protein.

Finally, histopathological analyses of the lungs of UG<sup>-/-</sup>, only, lacked mUG-specific immunostaining in bronchiolar epithelial cells. Lung tissues from UG<sup>-/-</sup>, UG<sup>+/-</sup> and UG<sup>+/+</sup> mice were fixed in Bouin's fluid or in 10% neutral buffered formalin fixatives, embedded in paraffin and sectioned at 4-6 microns. They were stained with hemotoxylin and eosin (H & E). Selected tissues were stained by Masson's trichrome method for collagen detection, PTAH for fibrin, or Congo Red for amyloid protein. For immunohistochemical detection of mUG and mFn, the Vectastain rabbit Elite ABC kit (Vector Laboratories) was used. The rabbit antibody (CytImmune) to mUG was raised by using a synthetic peptide (Peptide Technologies, Inc.) corresponding to mUG amino acid sequence (Lys28 to Thr49, specifically KPFNPGSDLQNAGTQLKRLVDT). The rabbit antibody to mFn (GIBCO BRL) was used at a dilution of 1:1000, and the antibody to mUG was used at 1:500.

These three sets of results confirm that the homozygous uteroglobin knockout mouse, UG<sup>-/-</sup>, lacks mUG protein, or any detectable piece of the protein.

#### **Example 5: Phenotype of uteroglobin knockout mouse**

Of the 179 mice born to crosses of UG<sup>+/+</sup> mice, 46 (26%) were of the +/+, 90 (50%) of the +/- and 43 (24%) of the UG<sup>-/-</sup> genotype, indicating that the disrupted mUG locus is inherited in a Mendelian fashion and that UG<sup>+/+</sup>, UG<sup>+/-</sup>, and UG<sup>-/-</sup> mice

were equally viable at birth. However,  $UG^{-/-}$  mice exhibited a novel phenotype in which they developed a progressive illness characterized by cachexia, heavy proteinuria, and hypocalcemia associated with profound weight loss. Proteinuria is a condition in which abnormally high levels of albumin and other serum proteins are excreted in the urine. It is indicative of glomerular dysfunction and renal failure. Histopathological examination of the kidneys of affected animals (as described above for lungs) revealed the fulminant renal glomerular disease shown in Figure 3. Compared with the glomeruli of the  $UG^{+/+}$  mice, those of  $UG^{-/-}$  mice were hypocellular and had massive eosinophilic proteinaceous deposits. The time course of the fatal renal disease in  $UG^{-/-}$  mice was either early onset (4-5 week period) or late onset (10 month period). Those  $UG^{-/-}$  mice that initially appeared healthy at 4 weeks of age had focal glomerular deposits at two months of age. At about 10 months, these mice had extreme cachexia similar to that of the mice dying of early onset disease. Heterozygotes had a milder form of the renal disease observed in  $UG^{-/-}$  mice. Histopathology of the kidneys of mice with late onset disease showed not only severe glomerulopathy as in the early onset disease, but also had marked fibrosis of the renal parenchyma and tubular hyperplasia (see Figure 3). Although the predominant pathology in the  $UG^{-/-}$  mice was found in the kidneys, histopathological studies also uncovered occasional focal areas of necrosis in the pancreas which appeared to be vascular oriented. Moreover, focal areas in the thymus and in the spleen structures suggestive of apoptotic bodies were also found. Interestingly, the pancreas expresses the mUG gene, and this organ is also a rich source of group-I extracellular PLA<sub>2</sub>; since this is primarily a digestive enzyme, its activation may cause tissue injury.

Because uteroglobin proteins have been reported to have immunomodulatory and anti-inflammatory properties and because reactive amyloidosis is known to occur in response to inflammation, it was likely that the glomerular deposits in the mUG-null mice were amyloid proteins. Reactive amyloidosis is characterized by the deposition of amyloid protein and immune complexes. The identity of the renal deposits in the  $UG^{-/-}$  mice was established by immunohistochemistry of kidney sections. Kidney sections from  $UG^{-/-}$  and  $UG^{+/+}$



mice were stained with Congo red and examined under the polarized light. Amyloid proteins yield a positive birefringence in this test; however, the glomeruli of  $UG^{-/-}$  mice were clearly negative. Immunofluorescence studies for the presence of IgA, IgG or IgM-immunocomplexes in the glomeruli of  $UG^{-/-}$  mice and immunohistochemical analyses for the presence of major amyloid proteins were also negative. Thus, the glomerular deposits of  $UG^{-/-}$  mice contained neither amyloid proteins nor immunocomplexes, and therefore, do not appear to be the result of an inflammatory response.

#### 10 Example 6: Detection of Fn and Collagen in $UG^{-/-}$ kidneys

The kidney deposits of  $UG^{-/-}$  mice were examined by transmission electron microscopy to elucidate their structure and morphology. A kidney from a  $UG^{-/-}$  mouse, with glomerular lesion, was fixed in formalin and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate for examination under the electron microscope. Photomicrographs were taken either at 6000X or at 60,000X. The deposits contained primarily two types of fibrillar structures: one type of long and striated fibrils which are relatively infrequent, the other short and diffuse which are more abundant (Figures 3E and 3F). Because ECM proteins, such as collagen and fibronectin, produce similar fibrillar structures, the glomerular deposits in  $UG^{-/-}$  mice may contain these proteins.

The glomerular deposits were next analyzed by immunofluorescence using anti-mFn antibody. Formalin-fixed tissue sections were used for immunofluorescence using a rabbit anti-mFn and FITC-conjugated goat anti-rabbit IgG. Similarly, immunofluorescence studies using antibodies specific for mFn, collagen I and III, vitronectin, laminin and osteopontin were also done. Epifluorescence was photographed using a Zeiss Axiophot microscope. Fn-specific immunofluorescence in the renal glomeruli of wild-type mice was virtually undetectable (Figure 3G), that in the glomeruli of  $UG^{-/-}$  littermates was intense (Figure 3H). When Masson's trichrome staining was used, the glomeruli of  $UG^{+/+}$  mice were negative (Figure 3I) and those of  $UG^{-/-}$  (Figure 3J) mice were positive, suggesting the presence of collagen

in the glomerular deposits. Immunofluorescence, using collagen I and collagen III-specific antibodies confirmed these results. Because Fn is known to interact with other extracellular matrix (ECM) proteins, we also tested for the presence of laminin, vitronectin and osteopontin in the glomeruli of UG<sup>+/+</sup> and UG<sup>-/-</sup> mice by  
5 immunohistochemistry, the results of which were negative.

**Example 7: Kidneys of UG<sup>-/-</sup> mice do not overproduce Fn**

In order to determine whether excessive production of Fn may account for its deposition in the renal glomeruli, we assessed the relative amount of Fn-mRNA in  
10 the kidneys, lungs, and the liver of UG<sup>-/-</sup> and UG<sup>+/+</sup> mice by RT-PCR and densitometry. The results indicate that relative amounts of Fn-mRNA were essentially identical in both UG<sup>+/+</sup> and UG<sup>-/-</sup> animals. Thus, over-production of Fn-mRNA was not a likely cause of Fn-deposition in the glomeruli of UG<sup>-/-</sup> mice. We then compared the Fn-protein in the plasma, kidneys, and the liver of UG<sup>-/-</sup> and UG<sup>+/+</sup>  
15 mice by SDS-PAGE under reducing conditions, and Western blotting. In the plasma, kidneys and the liver of wild-type mice only 220-kD Fn species could be detected; however, whereas the plasma and the liver lysate of UG<sup>-/-</sup> mice had the 220-kD Fn band, the kidney lysates contained another distinct, covalently linked, multimeric Fn-band (Figure 4A).

20

**Example 8: Elevated serum PLA<sub>2</sub> activity in UG<sup>-/-</sup> mice**

Based upon current concepts, critical initial steps in Fn matrix-assembly and fibrillogenesis, at least on the cell surface, are thought to involve integrin activation and Fn self-aggregation. Because UG is a potent inhibitor of soluble phospholipase  
25 A<sub>2</sub> (sPLA<sub>2</sub>), a key enzyme in the inflammatory pathway, the lack of mUG in UG<sup>-/-</sup> mice may contribute to the development of glomerulonephritis, an inflammatory renal disease. Thus, we measured PLA<sub>2</sub> activity in the serum of age, sex and weight-matched UG<sup>+/+</sup> (n=3) and UG<sup>-/-</sup> mice (n=3). The animals were sacrificed and serum PLA<sub>2</sub> activities of each sample were measured in triplicate using a PLA<sub>2</sub>-assay kit  
30 (Caymen Chemical) according to the instructions of the manufacturer. Protein

concentrations in the sera were determined by Bradford assay (Bio Rad) and specific activities of PLA<sub>2</sub> were calculated. The specific activities ( $\mu\text{mol}/\text{min}/\text{mg}$  protein) of serum PLA<sub>2</sub> of UG<sup>-/-</sup> mice [36 $\pm$  3.3 (SEM)] were significantly higher ( $p < 0.05$ ) than those of UG<sup>+/+</sup> mice [18 $\pm$  2.8 (SEM)]. These results raised the possibility that higher  
5 PLA<sub>2</sub> activity may lead to increased lysophosphatidic acid (LPA) production and consequently promote integrin activation and Fn-self aggregation in UG<sup>-/-</sup> mice.

**Example 9: Interaction of uteroglobin and fibronectin *in vitro***

To further understand how uteroglobin may prevent Fn self assembly, the  
10 ability of rhUG to disrupt mFn-Fn interaction *in vitro* was determined. Equimolar concentrations of rhUG and mFn were incubated to allow any protein binding or other interactions, then immunoprecipitated with anti-Fn-antibody, and the immunoprecipitates were resolved by SDS-PAGE under reducing conditions. Western blotting, as previously described, with either mFn or mUG antibody detected each  
15 protein, respectively. The results show that fibronectin co-immunoprecipitated with rhUG (Figure 4B). To confirm these results, the <sup>125</sup>I-rhUG was incubated with mFn and the complexes resolved by electrophoresis, using a 6% polyacrylamide gel under non-denaturing and non-reducing conditions (Figure 4C). Detection of a Fn-UG heteromer in the autoradiogram (lane 2) showed that soluble Fn interacts with UG *in*  
20 *vitro*. To ascertain whether Fn-UG heteromerization takes place *in vivo*, plasma of UG<sup>+/+</sup> and UG<sup>-/-</sup> mice was immunoprecipitated with an anti-mFn antibody that does not crossreact with rhUG (Figure 4D). Anti-mFn antibody co-precipitated both mFn and rhUG from the plasma of UG<sup>+/+</sup>, but not from UG<sup>-/-</sup> mice, suggesting that Fn-UG heteromers are present in the plasma of UG<sup>+/+</sup> mice. Therefore, the Fn-UG complex  
25 is not simply an artifact formed *in vitro* but occurs naturally in the serum.

To determine the specificity and affinity of UG binding to Fn, we incubated <sup>125</sup>I-Fn with unlabeled Fn in the presence and absence of UG. Any complexes were affinity-crosslinked with disuccinimidyl suberate (DSS). Using 24-well plates coated with human Fn (hFn) (Collaborative Biomedical Products), 3  $\mu\text{l}$  of <sup>125</sup>I-Fn (Sp. Act.  
30 6 mCi/mg; ICN Biomedicals) was incubated in the absence and presence of either UG

or Fn ( $10^{-12}$ - $10^{-6}$  M) in 500  $\mu$ l HBSS at room temperature for 2 hr. SDS-PAGE and Western blotting of all Fn with UG antibody failed to detect any UG contamination. The radiolabeled complex was washed twice with PBS, solubilized in 1 N NaOH, neutralized with 1 N HCl, and radioactivity was measured by a gamma counter. In  
5 a separate experiment  $^{125}$ I-hFn (3  $\mu$ l) was incubated with 20  $\mu$ l (1 mg/ml) of mouse Fn in 40  $\mu$ l of HBSS, pH 7.6 in the absence or presence of increasing concentrations of reduced rhUG (5-500  $\mu$ g) at room temperature for 2 hours. The samples were crosslinked with 0.20 mM DSS at room temperature for 20 min., boiled in SDS-sample buffer for 5 min., electrophoresed on 4-20% SDS-polyacrylamide gel and  
10 autoradiographed. In the absence of UG,  $^{125}$ I-Fn formed a high molecular weight, radioactive complex with unlabeled Fn, but in the presence of UG the formation of Fn-Fn aggregates was inhibited in a manner dependent upon the UG concentration (Figure 4E).

To determine whether there is any difference between the binding affinities of  
15 Fn for UG and that of Fn for itself binding experiments were performed in which  $^{125}$ I-Fn was incubated with unlabeled Fn and immobilized on multiwell plates together with varying concentrations of UG. In separate experiments, binding studies of  $^{125}$ I-Fn with unlabeled, immobilized Fn using various concentrations of unlabeled soluble Fn, were also done. The Scatchard analyses of the data from both types of binding  
20 experiments yielded straight lines with dissociation constants (kds) of 13 nM for UG binding to Fn and 176 nM for Fn binding to itself. These results suggest that, due to a relatively higher binding-affinity of UG for Fn, UG effectively counteracts Fn self-aggregation. Affinity-crosslinking experiments in which radio-iodinated ( $^{125}$ I)-collagen I was incubated with unlabeled Fn in the absence or presence of UG, were  
25 also done as described above for Fn. Fifteen  $\mu$ l of either denatured or non-denatured  $^{125}$ I-collagen I (Sp. Act. 65.4 mCi/mg) were incubated with Fn in presence or absence of reduced UG (250  $\mu$ g), affinity crosslinked, electrophoresed and autoradiographed. The results indicate that UG counteracts the formation of high molecular weight  $^{125}$ I-collagen-Fn aggregates (Figure 4F).

**Example 10: *In vivo* inhibition of glomerular hFn deposition by rhUG**

To test whether rhUG protects the renal glomeruli from Fn accumulation, soluble human Fn (hFn) alone, or hFn mixed with equimolar concentrations of rhUG, was administered intravenously to UG<sup>+/+</sup> and to apparently healthy UG<sup>-/-</sup> littermates.

5 Human Fn (500 µg/150 µl PBS) was administered in the tail vein of two-month old, approximately 22 g, UG<sup>+/+</sup> and apparently healthy, UG<sup>-/-</sup> mice. Similarly, the control mice were injected with a mixture of 500 µg of hFn either with equimolar concentrations of rhUG or albumin in 150 µl PBS. Twenty-four hours after the last injection, the mice were sacrificed and various organs were fixed in buffered  
10 formalin. The histological sections of the kidneys and other organs were examined by immunofluorescence with a monospecific anti-hFn antibody (GIBCO BRL; clone 1) and FITC conjugated rabbit anti-mouse IgG (Cappel). In a separate experiment, UG<sup>+/+</sup> mice were injected with 1 mg of hFn alone in 150 µl PBS daily for 3 consecutive days.

15 The rationale for injecting human Fn was to be able to discriminate between endogenous murine Fn and the administered hFn. The method of intravenous administration and immunohistochemical detection of hFn in various tissues have been described.

Human Fn immunofluorescence in the glomeruli of wild-type UG<sup>+/+</sup> mice  
20 injected with either a mixture of hFn and rhUG (1:1 molar ratio) or with hFn alone was similar (Figures 5A and 5B). However, the UG<sup>-/-</sup> mice injected with a mixture of hFn and UG showed little hFn-specific immunofluorescence in the glomeruli (Figure 5C), while those receiving Fn alone exhibited higher intensity immunofluorescence (Figure 5D). Administration of a mixture of hFn and BSA, as  
25 a control, yielded no protective effect.

To determine whether this UG protective effect could be overcome by injecting larger quantities of Fn in UG<sup>+/+</sup> mice, we injected 1 mg of hFn per animal daily for three consecutive days. Although intravenous administration of hFn to UG<sup>+/+</sup> mice at lower doses (500 µg/animal) was not effective in causing any  
30 appreciable glomerular deposition (Figure 5A), the administration of higher doses (3

mg/animal) led to a significant accumulation. Thus, UG prevents glomerular Fn-deposition, and UG<sup>+/+</sup> as opposed to UG<sup>-</sup> mice have a higher threshold for the accumulation of soluble Fn, due to the presence of endogenous UG.

**5 Example 11: Inhibition of fibrillogenesis and Fn matrix assembly by rhUG in tissue culture cells**

To determine whether UG prevents Fn-fibrillogenesis and matrix assembly in a typical *in vitro* tissue culture assay, mouse embryonic fibroblasts were cultured  
10 in medium containing either soluble hFn alone or a mixture of equimolar concentrations of hFn and rhUG. Fn matrix assembly and fibrillogenesis in cultured cells (CRL6336, ATCC) were determined as described. The level of fibrillogenesis seen in the cells of cultures treated with hFn alone was much higher (Figure 5E) compared to those which received a mixture of hFn and rhUG (Figure 5F).

15

**Example 12: Detection of UG-Fn complexes in clinical samples**

Detection of UG-Fn complexes in clinical samples of bodily fluids such as serum, BAL fluids, and sputum is important in determining the role of this complex in human disease. A solution phase diagnostic assay for the detection of UG-Fn  
20 complexes is developed and the assay format is shown in Figure 6. The capture antibody, covalently linked to a solid support, is a monospecific rabbit polyclonal raised against the human protein. The solid support may be a bead, such as a magnetic bead, a tube, or an ELISA plate. The solid support affords the flexibility of performing wash steps after each binding reaction in order to obtain more  
25 consistent results with a variety of sample types. The detection antibody is specific for Fn, and available from a number of commercial sources. An anti-IgG antibody, conjugated to an enzyme such as horse radish peroxidase (HRP), is then used to detect the anti-Fn IgG at the end of the molecular chain in a standard enzymatic reaction in which the enzyme substrate is converted to a chromogenic or fluorogenic  
30 compound that is quantitated with a spectrophotometer or fluorimeter (Amersham).

The detection limit for this assay is 500  $\mu$ g of UG-Fn complex per ml of sample fluid.

**Example 13: Uteroglobulin Deficiencies**

5        A transient but acute deficiency of hUG is created by the blood-cleansing technique known as clinical dialysis, including hemodialysis, peritoneal dialysis and continuous dialysis (CRRT). All forms of clinical dialysis involve the use of a semi-permeable membrane to filter toxic bodily waste products, including chemical metabolites such as urea, and small proteins such as beta2-microglobulin, out of the  
10    blood.

UG is an extremely compact protein, known for its anomolous migration in SDS-PAGE, corresponding to a molecular weight of approximately 10-13 kDa, despite its true molecular weight of 15.7 kDa. Therefore, the UG dimer was expected to behave as a 10-13 kDa protein in dialysis experiments. Surprisingly, it  
15    was found that the dimer is so compact that it passed through an 8.0 kDa MWCO dialysis membrane. UG also passed through a 14.0 kDa MWCO dialysis membrane.

The composition of the dialysis membranes used in these examples are similar, if not identical, to the composition of the majority of membranes manufactured and used for clinical dialysis. They consist of regenerated cellulose or cellulose acetate.

20        For this experiment, 1.0 ml aliquots of two partially purified (one >90% pure and one approximately 70% pure) rhUG cell lysates, with no buffer additives, were dialyzed against 1000 mls of unbuffered 50 mM ammonium acetate, using three sizes of dialysis tubing: 3.5 kDa, 8.0 kDa, and 14.0 kDa (Spectra/Por; Thomas Scientific). There were four changes of buffer for each sample over a 48 hour time  
25    period, all done at room temperature (about 25-27°C). The appearance of each dialysis sample changed from a clear yellow to a clear, colorless liquid. Dialysis tubing was checked for leaks at the beginning and end of the process by brief application of pressure directly to the tubing (squeezing) and observation of any leaks, of which there were none. Tubing was double clamped at either end to further insure  
30    against leaks.

Figure 7 shows the SDS-PAGE analysis of these results. The 90% pure pre-dialysis sample is shown in lane 7 and 8 next to the three post-dialysis samples in lanes 1, 2, and 3. The UG dimer is no longer present in the lanes representing the samples dialysed with 8.0 kDa MWCO membranes. These results were later  
5 confirmed with different batches of partially purified UG preparations.

While the invention has been described in connection with what is presently considered to be the most practical and preferred embodiments, it is to be understood that the invention is not to be limited to the disclosed embodiments but, on the  
10 contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.



**REFERENCES**

1. Levin, S.W. *et al.*, Life Sci. 38: 1813-1819 (1986);
2. Singh G. *et al.*, Biochem. Biophys. Acta. 1039: 348-355(1990);
3. Mantile, G. *et al.*, J. Biol. Chem 268: 20343-20351 (1993);
- 5 4. Singh, G. *et al.*, J. Histochem. Cytochem. 36: 73-80 (1987);
5. Bernard, A. *et al.*, Clin. Chem. 38: 434-435 (1992);
6. Dhanireddy, R. *et al.*, Pediatric Res. 23: 463A (1988);
- 10 7. Dhanireddy, R. *et al.*, Pediatric Res. 33: 323A (1993);
8. Piomelli, D., Op. In Cell Biol. 5: 274-280(1993);
9. Krishnan, R.S. *et al.*, Science 158: 490-492 (1967);
- 15 10. Beier, H. Verhandl Deut. Zool. Ges. Heidelberg (1968);
11. Umland, T.C. *et al.*, Nature Stuct. Biol. 1: 538-545 (1994);
- 20 12. Hard, T. *et al.*, Nature. Struct. Biol. 2: 938-989 (1995);
13. Umland, T.C. *et al.*, Nature Struct. Biol. 2: 919-922(1995);
14. Stripp, B. R. *et al.*, Am. T. Physio. 271 (Lung Cell. Mol. Physiol. 15):  
25 L656-L664 (1996);
15. Lesur, O. *et al.*, Am. T. Respir. Crit. Care Med. 152: 290-297 (1995);
16. Glaser, K.B., Adv. Pharmacol. 32: 31-66 (1995);
- 30 17. Tykka, H.T. *et al.*, Scand. J. Gastroenterol. 20: 5-12 (1985);
18. Sheuer, W., Klin. Wochenschr. 67: 153-159 (1989);
- 35 19. Barnes, H.J. *et al.*, J. Mol. Biol., Feb. 23, 1996;
20. Aoki, A. *et al.*, Mol. Hum. Reprod. 2: 419-497 (1996);
21. Anderson and Kurkland, Microbiological Reviews 54: 198-210 (1990);
- 40

22. Miele, L. *et al.*, J. Biol. Chem. 265: 6427-6435 (1990);
23. Coalson, J.J. *et al.*, Exp. Mol. Pathol. 37: 355-360 (1982);
- 5 24. Nagy, A. *et al.*, Proc. Natl. Acad. Sci. 90: 8424 (1993);
25. Capecchi, M.R., Science, 244: 1288 (1989);
- 10 26. Harlow, E. and Lane D. Antibodies: A Laboratory Manual, 1st Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988;
27. Mantile, G. *et al.*, J. Biol. Chem. 267: 20343 (1993);
- 15 28. Ruoslahti, E. Ann Rev. Biochem. 57: 375 (1988);
29. R.O. Hynes, Fibronectins, New York: Springer-Verlag (1990);
30. Chernousor, M.A. *et al.*, J. Biol. Chem. 266: 10857 (1991);
- 20 31. Zhang, Q. *et al.*, J. Cell. Biol. 127: 1447 (1994);
32. Wu, C. *et al.* Cell 83: 715 (1995);
33. Zhang, Q. *et al.*, J. Biol. Chem. 271: 33284 (1996);
- 25 34. Border, W.A. *et al.*, J. Clin. Invest. 90: 1 (1992);
35. Peri, A., *et al.*, J. Clin. Invest. 92: 2099 (1995);
- 30 36. Peri, A. *et al.*, J. Clin. Invest. 96: 343 (1995);
37. Oh, E. *et al.*, Proc. Natl. Acad. Sci. (USA) 78: 3218 (1981);
38. Mosher, D.F. *et al.*, Curr, Biol. 4: 810 (1992).

**WHAT IS CLAIMED IS:**

1. A pharmaceutical composition comprising a PLA<sub>2</sub> inhibiting effective  
5 amount of recombinant human uteroglobin (rhUG) or a fragment or a derivative thereof and a pharmaceutically acceptable carrier or diluent.
2. The pharmaceutical composition of claim 1 further comprising a lung surfactant.
3. The pharmaceutical composition of claim 1 wherein said rhUG has a  
10 purity of about 75% to about 100%.
4. The pharmaceutical composition of claim 1 wherein said rhUG has a purity of about 90% to about 100%.
5. The pharmaceutical composition of claim 1 wherein said rhUG has a purity of at least 95%.
- 15 6. A pharmaceutical composition comprising a fibronectin binding effective amount of recombinant human uteroglobin (rhUG) or a fragment or a derivative thereof and a pharmaceutically acceptable carrier or diluent.
7. The pharmaceutical composition of claim 6 further comprising a lung surfactant.
- 20 8. The pharmaceutical composition of claim 6 wherein said rhUG has a purity of about 75% to about 100%.
9. The pharmaceutical composition of claim 6 wherein said rhUG has a purity of about 90% to about 100%.
10. The pharmaceutical composition of claim 6 wherein said rhUG has a  
25 purity of at least 95%.
11. A pharmaceutical composition comprising a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of UG in admixture with an active agent for treatment of a target indication.
12. The pharmaceutical composition of claim 11, wherein said PLA<sub>2</sub>  
30 inhibiting or fibronectin binding effective amount of rhUG reduces inflammation and

thereby insures that an effective amount of said active agent reaches the treatment site.

13. A method for inhibiting PLA<sub>2</sub> enzymes *in vivo* in a mammal in need of such treatment, said method comprising administering to said mammal a PLA<sub>2</sub> inhibiting effective amount of native or recombinant human uteroglobin (UG) or a  
5 fragment or derivative thereof.

14. The method of claim 13 wherein said UG has a purity of between about 75% to about 100%.

15. The method of claim 13 wherein said UG has a purity of between about  
10 90% to about 100%.

16. The method of claim 13 wherein said UG has a purity of at least 95%.

17. The method of claim 13 wherein said UG is recombinant (rhUG), having substantially the same sequence as native human uteroglobin.

18. The method of claim 13 wherein said PLA<sub>2</sub> inhibiting effective amount  
15 of UG is 20ng/kg to 500 mg/kg.

19. The method of claim 13 wherein said UG is administered in association with a lung surfactant.

20. The method of claim 19 wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

21. The method of claim 13 wherein said UG is administered by the  
20 endotracheal, ophthalmic, intravenous, systemic, intraperitoneal, intramuscular, or oral routes.

22. The method of claim 13 wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.

23. The method of claim 13 wherein said method is for the treatment of  
25 a condition selected from the group consisting of systemic inflammation, asthma, cystic fibrosis, ocular inflammation, premature labor, infertility, rheumatoid arthritis, type 1 diabetes, nephropathy, inflammatory bowel disease, Crohn's disease, ulcerative colitis, pancreatitis, peritonitis, allergy, multiple organ failure, ARDS, acute renal

failure, inflammation secondary to infection or surgery, and transplanted organ rejection.

24. The method of claims 23 wherein ocular inflammation results from autoimmune uveitis and corneal transplant surgery.

5 25. The method of claim 13 wherein said method is for the treatment of inflammation and immune modulation.

26. A method for treating or preventing an inflammatory condition in a patient in need of such treatment, said method comprising administering to said patient an anti-inflammatory effective amount of native or recombinant UG or a  
10 fragment or derivative thereof.

27. The method of claim 26 wherein said UG has a purity of between about 75 % to about 100%.

28. The method of claim 26 wherein said UG has a purity of between about 90 % to about 100%.

15 29. The method of claim 26 wherein said UG has a purity of at least 95 %.

30. The method of claim 26 wherein said UG is recombinant (rhUG) and has substantially the same sequence as the native human uteroglobin.

31. The method of claim 26 wherein said  $\text{PLA}_2$  inhibiting effective amount of UG is 20ng/kg to 500 mg/kg.

20 32. The method of claim 26 wherein said UG is administered in association with a lung surfactant.

33. The method of claim 32 wherein said lung surfactant is present in an amount of about 20 % to about 80 % by weight.

34. The method of claim 26 wherein said UG is administered by the  
25 endotracheal, ophthalmic, intravenous, systemic, intraperitoneal, intramuscular, or oral routes.

35. The method of claim 26, wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.

36. The method of claim 26, wherein said inflammatory condition is  
30 neonatal RDS.

37. The method of claim 26 wherein said inflammatory condition is adult RDS.

38. The method of claim 26, wherein said inflammatory condition is caused by a viral infection, bacterial infection, parasitic infection, or fungal infection.

5 39. The method of claim 26, wherein said method is for the treatment of an inflammatory condition selected from the group consisting of systemic inflammation, asthma, cystic fibrosis, ocular inflammation, premature labor, remote organ failure, rheumatoid arthritis, pancreatitis, septic shock, collagen vascular disease, anaphylactic shock, trauma-induced shock, acute renal failure, cystitis,  
10 inflammation of the urethra, inflammation of the ureter, bladder inflammation, interstitial cystitis, vaginitis, inflamed cervix, pelvic inflammatory disease, inflammation of the ovariosalpingitis, inflammation of the fallopian tubes, penile inflammation, prostate inflammation, inflammation of seminal tubules and seminal vesicles, testicular inflammation, inflammation of vas deferens, epididymis, and  
15 prostate gland, retinitis, burn damage due to fire or chemical burn, bronchial asthma, ARDS, neonatal RDS, directiculitis, neonatal necrotizing enterocolitis, stomach ulcers, gastrointestinal reflux disease, tonsillitis, hemorrhoids, otitis media, psoriasis, hives, allergic dermatitis, contact dermatitis, chemical dermatitis, endocarditis, and infertility.

20 40. The method of claim 39, wherein said inflammation is ocular inflammation that results from autoimmune uveitis, CMV retinitis, bacterial infection, viral infection, parasitic infection, the presence of an infectious agent, retinoblastoma, radiation exposure, allergic response, and corneal transplant surgery.

41. A method for treating or preventing a fibrotic condition in a patient in  
25 need of such treatment, said method comprising the step of administering to a patient a fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

42. The method of claim 41, wherein said UG has a purity of between about 75% to about 100%.

43. The method of claim 41, wherein said UG has a purity of between about 90% to about 100%.

44. The method of claim 41, wherein said UG has a purity of at least 95%.

45. The method of claim 41, wherein said UG is recombinant (rhUG) and  
5 has substantially the same sequence as native human uteroglobin.

46. The method of claim 41, wherein said fibronectin binding effective amount of UG is 20ng/kg to 500 mg/kg.

47. The method of claim 41, wherein said UG is administered in association with a lung surfactant.

10 48. The method of claim 47, wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

49. The method of claim 41, wherein said UG is administered by the endotracheal, ophthalmic, intravenous, intraperitoneal, intramuscular, liquid or oral routes.

15 50. The method of claim 41, wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.

51. The method of claim 41, wherein said fibrotic condition is pulmonary fibrosis.

52. The method of claim 41, wherein said fibrotic condition is renal  
20 fibrosis.

53. The method of claim 41, wherein said fibrotic condition is vascular fibrosis.

54. A method for treating or preventing an inflammatory or fibrotic condition characterized by a deficiency of endogenous UG, said method comprising  
25 administering to a patient in need of such treatment a compensating amount of native or recombinant UG or a fragment or derivative thereof.

55. The method of claim 54, wherein said UG has a purity of between about 75% to about 100%.

56. The method of claim 54, wherein said UG has a purity of between  
30 about 90% to about 100%.

57. The method of claim 54, wherein said UG has a purity of at least 95%.

58. The method of claim 54, wherein said UG is recombinant (rhUG) and has substantially the same sequence as native human uteroglobin.

59. The method of claim 54, wherein said compensating amount UG is  
5 20ng/kg to 500 mg/kg.

60. The method of claim 54, wherein said UG is administered in association with a lung surfactant.

61. The method of claim 60, wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

10 62. The method of claim 54, wherein said UG is administered by the endotracheal, ophthalmic, intravenous, systemic, intraperitoneal, intramuscular, or oral routes.

63. The method of claim 54, wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.

15 64. The method of claim 54, wherein said method is for the treatment of an inflammatory or fibrotic condition characterized by a deficiency of endogenous UG, wherein said condition is selected from the group consisting of neonatal bronchopulmonary dysplasia, complications of hemodialysis, bleomycin lung chronic obstructive pulmonary disease, and inherited glomerulopathy.

20 65. The method of claim 26, wherein UG is administered in combination with an active agent selected from the group consisting of steroids, nonsteroidal antiinflammatory agents, chemotherapeutics, analgesics, antibodies, antiparasites, antivirals, antibiotics, antifungals, vaccines, tumor vaccines, immunosuppressives, hematopoietic growth factors, antithrombotics, cardiovascular drugs, one or more  
25 vitamin and mineral supplements, and fertility drugs.

66. The method of claim 44, wherein UG is administered in combination with an active agent selected from the group consisting of steroids, nonsteroidal antiinflammatory agents, chemotherapeutics, analgesics, antibodies, antiparasites, antivirals, antibiotics, antifungals, vaccines, tumor vaccines, immunosuppressives,



growth factors, antithrombotics, cardiovascular drugs, one or more vitamin and mineral supplements, and fertility drugs.

67. The method of claim 54, wherein UG is administered in combination with an active agent selected from the group consisting of steroids, nonsteroidal  
5 antiinflammatory agents, chemotherapeutics, analgesics, antibodies, antiparasites, antivirals, antibiotics, antifungals, vaccines, tumor vaccines, immunosuppressives, growth factors, antithrombotics, cardiovascular drugs, one or more vitamin and mineral supplements, and fertility drugs.

68. A cosmetic composition comprising a  $PLA_2$  inhibiting effective amount  
10 of native or recombinant human uteroglobin (UG) or a fragment or derivative thereof and a pharmaceutically acceptable carrier or diluent.

69. The cosmetic composition of claim 68 further comprising a lung surfactant.

70. The cosmetic composition of claim 68 wherein said UG has a purity  
15 of about 75% to about 100%.

71. The cosmetic composition of claim 68 wherein said UG has a purity of about 90% to about 100%.

72. The cosmetic composition of claim 68 wherein said UG has a purity of at least 95%.

20 73. A cosmetic composition comprising a fibronectin binding effective amount of native or recombinant human uteroglobin (UG) or a fragment or derivative thereof, and an acceptable carrier or diluent.

74. The cosmetic composition of claim 73 further comprising a lung surfactant.

25 75. The cosmetic composition of claim 73 wherein said UG has a purity of about 75% to about 100%.

76. The cosmetic composition of claim 73 wherein said UG has a purity of about 90% to about 100%.

30 77. The cosmetic composition of claim 77 wherein said UG has a purity of at least 95%.

78. A blood supplement comprising a PLA<sub>2</sub> inhibiting effective amount of native or recombinant human uteroglobin (UG) or a derivative or fragment thereof, and a pharmaceutically acceptable carrier or diluent.

79. The blood supplement composition of claim 78 further comprising a  
5 lung surfactant.

80. The blood supplement composition of claim 78 wherein said UG has a purity of about 75% to about 100%.

81. The blood supplement composition of claim 78 wherein said UG has a purity of about 90% to about 100%.

10 82. The blood supplement composition of claim 78 wherein said UG has a purity of at least 95%.

83. A blood supplement composition comprising a fibronectin binding effective amount of native or recombinant human uteroglobin (UG) or a fragment or derivative thereof, and a pharmaceutically acceptable carrier or diluent.

15 84. The blood supplement composition of claim 83 further comprising a lung surfactant.

85. The blood supplement composition of claim 83 wherein said UG has a purity of about 75% to about 100%.

20 86. The blood supplement composition of claim 83 wherein said UG has a purity of about 90% to about 100%.

87. The blood supplement composition of claim 83 wherein said UG has a purity of at least 95%.

25 88. An assay for quantitating uteroglobin-fibronectin complexes in a clinical sample, wherein a clinical sample suspected of containing one or more uteroglobin-fibronectin complexes is

- (a) contacted with an antigen capture agent,
- (b) an antigen detection agent is added to said sample, and
- (c) the presence of any complex bound to said capture agent is detected using a secondary antibody conjugated to a moiety capable of being detected  
30 by the addition of a compound which reacts with said chemical moiety.

89. The assay of claim 88 wherein said antigen capture agent is a monospecific rabbit polyclonal antibody, immobilized on an insoluble support.

90. The assay of claim 88 wherein the secondary antibody is an anti-IgG antibody conjugated to an enzyme and the compound which reacts with the chemical moiety is a substrate for the enzyme which is converted to a chromogenic or fluorogenic compound upon reaction with the enzyme.

91. The assay of claim 90 wherein the enzyme is horse radish peroxidase.

92. The assay of claim 88 wherein the antigen detection agent is an antibody specific for fibronectin.

93. A method for treating or preventing fibrillogenesis in a patient in need of such treatment, said method comprising the step of administering to a patient a fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

94. The method of claim 93, wherein said UG has a purity of between about 75% to about 100%.

95. The method of claim 93, wherein said UG has a purity of between about 90% to about 100%.

96. The method of claim 93, wherein said UG has a purity of at least 95%.

97. The method of claim 93, wherein said UG is recombinant (rhUG) and has substantially the same sequence as native human uteroglobin.

98. The method of claim 97, wherein said fibronectin binding effective amount of UG is 20ng/kg to 500 mg/kg.

99. The method of claim 97, wherein said UG is administered in association with a lung surfactant.

100. The method of claim 99, wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

101. The method of claim 93, wherein said UG is administered by the endotracheal, ophthalmic, intravenous, intraperitoneal, intramuscular, liquid or oral routes.

102. The method of claim 93, wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.
103. The method of claim 93, wherein said fibrotic condition is pulmonary fibrosis.
- 5 104. The method of claim 93, wherein said fibrotic condition is renal fibrosis.
105. The method of claim 93, wherein said fibrotic condition is vascular fibrosis.
106. The method of claim 38 wherein said viral infection is CMV retinitis.
- 10 107. The method of claim 38 wherein said bacterial infection is pneumonia or cystitis.
108. The method of claim 38 wherein said parasitic infection is schistosomiasis.
109. The method of claim 38 wherein said fungal infection is vaginal  
15 candidiasis.

## FIG. 1

Human CC10 kDa: 1 EICPSFQRVIEITLLMDTPSSYEAAAMELFSPDDDMREAGAKKLVDTLPQKPREIIKLMKIAQSSLCN 70  
 ICP F VIE LL TPSSYE F PC M AG Q KK D LPQ RE I KL EKI S LC  
 Rabbit Utg: GICPRFAHVIEIENLLGTPSSYEISLKEFEPDDTMKDAQMCKKVLDSLPTTRENIMKLTKEIKVKSPLC

ICP F V E LL S YEAA F P D AG QLK LVDTL PQ R I KL EKI S LC  
 Rat CC10: DICPGFLQVLEALLGSESNYEAAALPKFNPASDLQNA GTQLKRLVDTL PQETRINIVKLT EKITLSP LCEQDLRV

ICP F V E LLM S Y A F P D AG QLK LVDTL PQ R I KL EKI S LC  
 Mouse CC10: DICPGFLQVLEALLMESESGYVASLKPFPNPGSD LQNA GTQLKRLVDTL PQETRINIMKLT EKITLSP LCKQDLRF

For comparison, amino acid residues are shown above the primary sequences correspond to residues found at the same position in the human protein. These sequences are predicted by translation of the nucleotide sequences deposited in the Genebank. These proteins are secreted proteins and the N-terminal signal sequences, that are translated and processed off, have been edited out for this comparison.

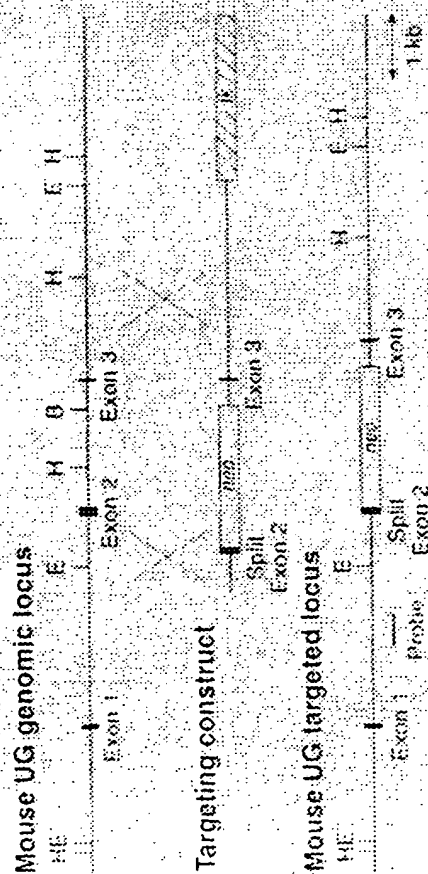


FIG. 2A

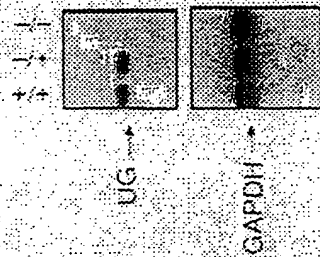


FIG. 2E

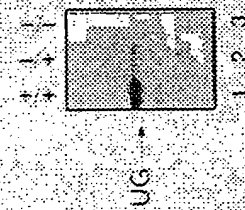


FIG. 2F



FIG. 2D

FIG. 2C

FIG. 2B

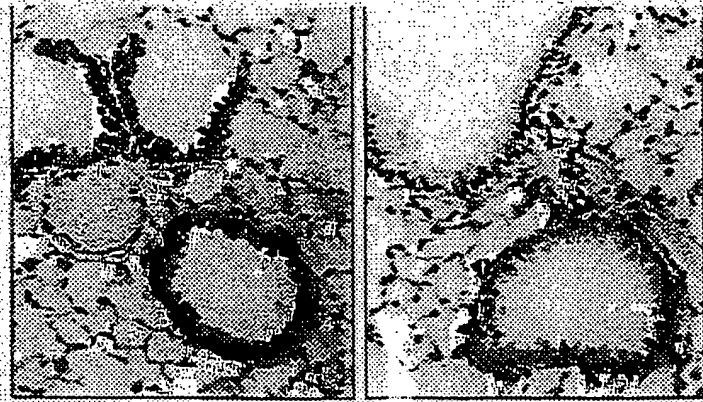


FIG. 2G



FIG. 3D

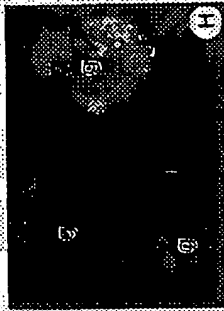


FIG. 3H

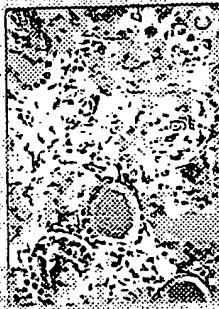


FIG. 3C

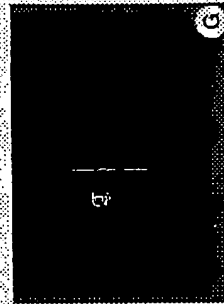


FIG. 3G



FIG. 3J

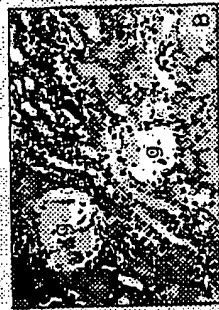


FIG. 3B



FIG. 3F



FIG. 3I



FIG. 3A



FIG. 3E

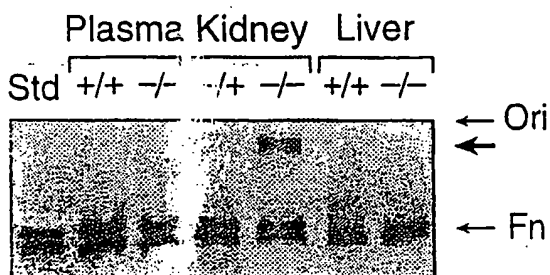


FIG. 4A

FIG. 4B

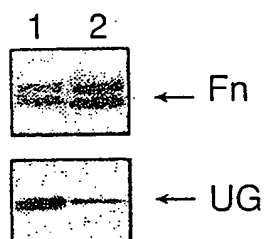


FIG. 4C

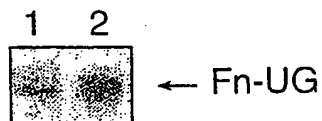


FIG. 4D

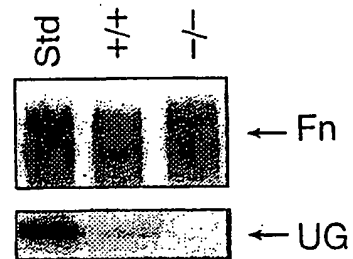


FIG. 4F

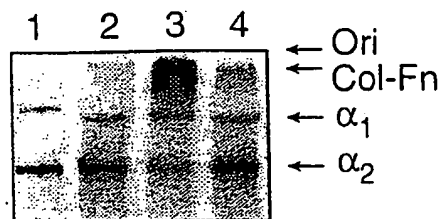
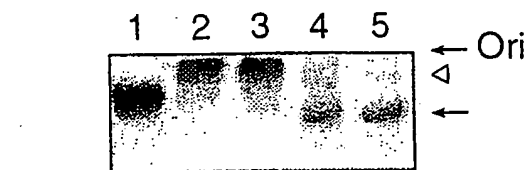


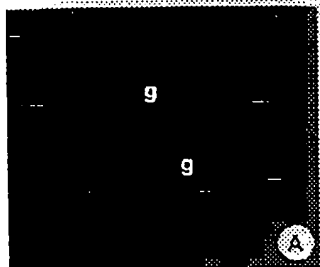
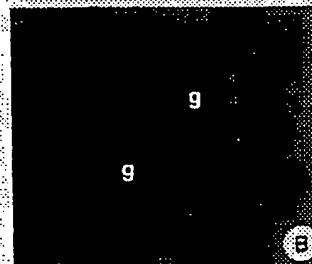
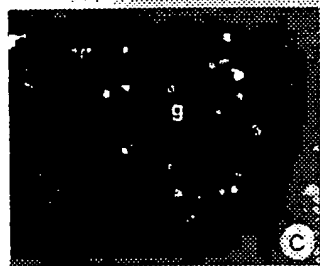
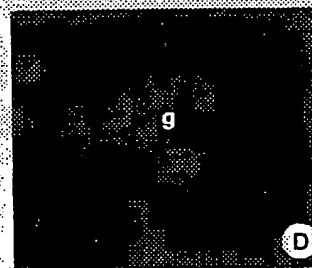
FIG. 4E

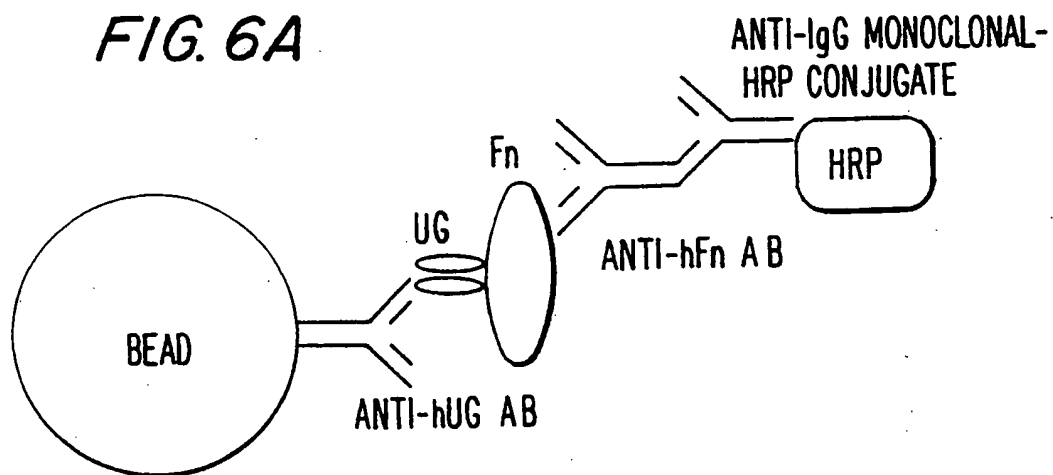
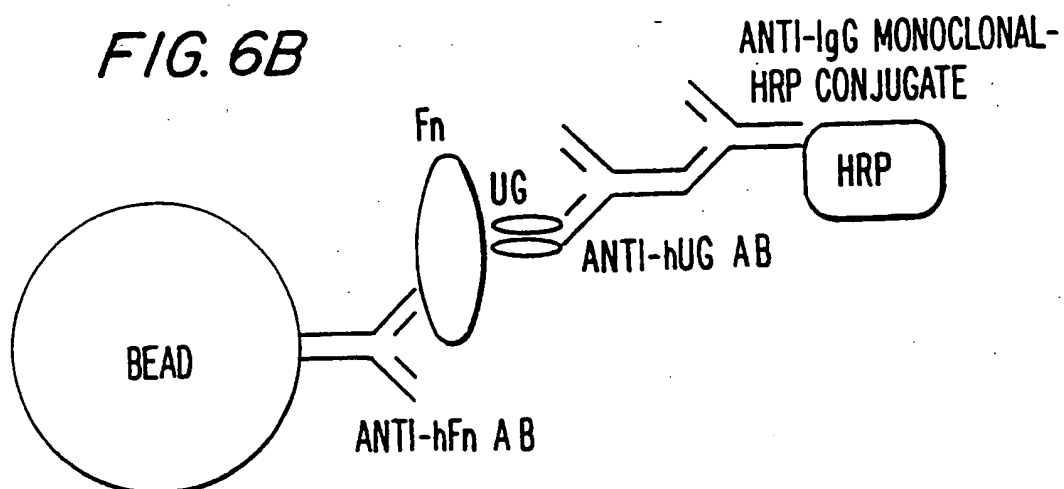


<sup>125</sup> I-Fn	+	+	+	+	+
Fn	+	+	+	+	+
UG	-	-	5μg	50μg	500μg
DSS	-	+	+	+	+

<sup>125</sup> I-Col I	+	+	+
Fn	+	+	+
UG	-	-	+
DSS	-	+	+



*FIG. 5A**FIG. 5B**FIG. 5C**FIG. 5D**FIG. 5E**FIG. 5F*

**FIG. 6A****FIG. 6B**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/11026

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/17; C07K 14/875

US CL : 514/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS

search terms: uteroglobin, phospholipase, fibronectin, lung surfactant

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	LEYTON et al. Recombinant human uteroglobin inhibits the in vitro invasiveness of human metastatic prostate tumor cells and the release of arachidonic acid stimulated by fibroblast-conditioned medium. Cancer Res. 15 July 1994, Vol. 54, pages 3696-3699, see Abstract and page 3697, column 1, full paragraph 1.	1, 3-6, 8-10 -- 2, 7, 11, 12
Y	EDELSON et al. Acute lung injury induced by phospholipase A <sub>2</sub> : Structural and functional changes. Am. Rev. Respir. Dis. MAY 1991, Vol. 143, pages 1102-1109, see page 1102, column 1, full paragraph 1, paragraphs bridging columns 1-2 and columns 2-3, paragraph bridging pages 1105-1106.	2, 7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

17 AUGUST 1998

Date of mailing of the international search report

24 SEP 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DAVID S. ROMEO

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11026

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,266,562 A (MUKHERJEE et al.) 30 November 1993, column 8, full paragraph 1.	11, 12

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/11026

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-12
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☒

No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11026

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-5, drawn to a pharmaceutical composition.

Group II, claim(s) 6-10, drawn to a pharmaceutical composition.

Group III, claim(s) 11-12, drawn to a pharmaceutical composition.

Group IV, claim(s) 13-25, drawn to a method of inhibiting  $PLA_2$  *in vivo*.

Group V, claim(s) 26-40, 65, and 106-109, drawn to a method of treating an inflammatory condition.

Group VI, claim(s) 41-53, and 66, drawn to a method of treating a fibrotic condition.

Group VII, claim(s) 54-64, and 67, drawn to a method of treating a uteroglobin deficiency.

Group VIII, claim(s) 68-72, drawn to a cosmetic composition.

Group IX, claim(s) 73-77, drawn to a cosmetic composition.

Group X, claim(s) 78-82, drawn to a blood supplement.

Group XI, claim(s) 83-87, drawn to a blood supplement.

Group XII, claim(s) 88-92, drawn to an assay for quantitating uteroglobin.

Group XIII, claim(s) 93-105, drawn to a method of treating fibrillogenesis.

The inventions listed as Groups I-XIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

With respect to unity of invention PCT Rule 13.1 states:

The international application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention").

Additionally, PCT Rule 13.2 states:

Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

With regard to the application of PCT Rule 13, 37 CFR § 1.475 concerning unity of invention states:

(a) An international and a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in an application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

(c) If an application contains claims to more or less than one of the combinations of categories of invention set forth in paragraph (b) of this section, unity of invention might not be present.

(d) If multiple products, processes of manufacture or uses are claimed, the first invention of the category first mentioned in the claims of the application and the first recited invention of each of the other categories related

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11026

thereto will be considered as the main invention in the claims, see PCT Article 17(3)(a) and 37 C.F.R. § 1.476(c).

(e) The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim.

Groups I-XIII recite a plurality of pharmaceutical compositions and/or methods each having a different intended use. The single inventive concept joining these disparate intended uses appears to be that the pharmaceutical compositions comprise and the methods use recombinant human uteroglobin. In order for the inventions of groups I-XIII to have unity of invention it is necessary that the single inventive concept be a contribution to the prior art. However, LEYTON et al. (Cancer Research 54:3696-3699, July 15, 1994) teach recombinant human uteroglobin, teach that 1  $\mu$ M recombinant human uteroglobin inhibits arachidonic acid release, and suggest recombinant human uteroglobin inhibits tumor cell invasiveness through inhibition of PLA<sub>2</sub>-mediated arachidonic acid release. A pharmaceutical composition comprising a PLA<sub>2</sub> inhibiting effective amount of recombinant human uteroglobin would have been obvious to one of ordinary skill in the art. Therefore, the inventions of groups I-XIII do not fulfill the requirements for unity of invention with respect to a contribution which each of the inventions makes over the prior art. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 38/17, C07K 14/875</b>		<b>A1</b>	(11) International Publication Number: <b>WO 98/53846</b>
			(43) International Publication Date: 3 December 1998 (03.12.98)
(21) International Application Number: PCT/US98/11026 (22) International Filing Date: 28 May 1998 (28.05.98) (30) Priority Data: 08/864,357          28 May 1997 (28.05.97)          US (71) Applicants: CLARAGEN, INC. [US/US]; 335 Paint Branch Road, College Park, MD 20742 (US). NATIONAL INSTITUTE OF HEALTH [US/US]; Dept. of Health and Human Services, Office of Technology Transfer, Suite 325, 6011 Executive Building, Rockville, MD 20852 (US). (72) Inventors: PILON, Aprile. L.; 11333 Rambling Road, Gaithersburg, MD 20879 (US). MUKHERJEE, Anil, B.; P.O. Box 136, Brookeville, MD 20833-0136 (US). ZHANG, Zhongjian; 10 Chantilly Court, Rockville, MD 20850 (US). (74) Agents: EVANS, Barry et al.; Whitman Breed Abbott & Morgan LLP, 200 Park Avenue, New York, NY 10166 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: USE OF RECOMBINANT HUMAN UTEROGLOBIN IN TREATMENT OF INFLAMMATORY AND FIBROTIC CONDITIONS			
(57) Abstract			
<p>Methods for treatment of inflammatory and fibrotic conditions <i>in vivo</i> using UG is disclosed. Methods for treating or preventing inflammatory or fibrotic conditions characterized by a deficiency of endogenous functional UG are also disclosed. Compositions containing UG, optionally containing lung surfactant, and assay procedures for detection of UG-fibronectin complexes, are also provided.</p>			



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## USE OF RECOMBINANT HUMAN UTEROGLOBIN IN TREATMENT OF INFLAMMATORY AND FIBROTIC CONDITIONS

### CROSS-REFERENCE TO RELATED APPLICATIONS

5

The present application is a continuation-in-part of U.S. Application Serial No. 08/864,357, the disclosure of which is incorporated herein by reference.

### FIELD OF THE INVENTION

10

The invention relates generally to the treatment of inflammatory and fibrotic conditions using native human uteroglobin (hUG) or recombinant human uteroglobin (rhUG). Novel physiological roles and therapies for UG (hUG or rhUG) have been identified. Specifically, the invention relates to the treatment of inflammatory and  
15 fibrotic conditions by administering hUG or rhUG to inhibit PLA<sub>2</sub>s and/or to prevent fibronectin deposition. The invention further provides a method for the treatment of neonatal respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD), a critical clinical condition of the lung, and glomerular nephropathy, a disease of the kidney, both characterized by the inflammatory and fibrotic conditions.

20

Documents cited in this application relate to the state-of-the-art to which this invention pertains, each of which is incorporated herein by reference.

### BACKGROUND OF THE INVENTION

#### Inflammatory and Fibrotic Conditions

25

The search for improved therapeutic agents for the treatment of inflammatory and fibrotic diseases has received much attention in recent years. Neonatal RDS, a lung surfactant deficiency disease, is a condition of particular interest in that it is one of the major causes of mortality in premature neonates. While introduction of surfactant therapy dramatically improves survival of RDS patients, the development  
30 of chronic inflammatory and fibrotic disease in a significant percentage of this patient population is a major problem. Likewise, hereditary fibronectin-deposit glomerular nephropathy leads to end stage renal failure when patients' kidneys become blocked

and no longer filter the blood. Nephropathy is characterized by fibronectin deposits and fibrosis of the kidneys which render the organ non-functional, and eventually, unable to support life.

PLA<sub>2</sub> (phospholipase A<sub>2</sub>), a class of endogenous enzymes that hydrolyze the  
5 Sn2 position ester bond of glycerophospholipids, is one of many proteins implicated in inflammatory and fibrotic conditions. It is also responsible for hydrolysis of surfactant phospholipids in the lungs. Uteroglobin (also known as CC10, CC16, CC17, urine protein-1, P-1, progesterone binding protein, PCB-binding protein, Clara cell secretory protein (CCSP), blastokinin, retinol-binding protein, phospholipid-  
10 binding protein, and alpha2-microglobulin) inhibits the activity of PLA<sub>2</sub> *in vitro*.

Uteroglobin is a small globular homodimeric protein. It has a molecular weight of 15.8 kDa, but it migrates in electrophoretic gels at a size corresponding to 10 kDa. Human uteroglobin is abundant in the adult human lung, and comprises up to about 7% of the total soluble protein. However, its expression is not fully  
15 activated in the developing human fetus until late in gestation. Consequently, the extracellular lung fluids of pre-term infants contain far less UG than those of adults. UG is also expressed by the pancreas.

PLA<sub>2</sub>s play critical roles in the inflammatory response because they release arachidonic acid (AA) from cellular phospholipid reservoirs. AA is metabolized to  
20 a number of potent inflammatory mediators in a process referred to as the arachidonic acid cascade.

Several acute and chronic clinical conditions have been characterized by elevated serum or local PLA<sub>2</sub> activity (see Table 1, below).

**Table 1. Clinical Conditions Associated with PLA<sub>2</sub> Activity**

	<u>Diseases</u>	<u>Sites</u>
	Rheumatoid arthritis	Serum, synovial fluid, WBC
5	Collagen vascular diseases	Serum
	Pancreatitis	Serum
	Peritonitis	Peritoneal fluid and cells
	Septic shock	Serum
	ARDS*	Serum and alveolar fluid
10	Acute renal failure	Serum
	Autoimmune uveitis	Serum, aqueous humor
	Bronchial asthma	Bronchial fluid

\* Adult respiratory distress syndrome

15

There are no effective PLA<sub>2</sub> inhibitors presently available for clinical use. To date, only a few PLA<sub>2</sub> inhibitors have progressed into clinical trials, but none have qualified for commercial marketing.

Fibronectin (Fn) is a 200 kDa glycoprotein which exists in several different forms and is secreted by different tissues. Fn is an essential protein and targeted disruption of the Fn gene in mice showed that it has a central role in embryogenesis. Fn also plays a key role in inflammation, cell adhesion, tissue repair and fibrosis, and is deposited at the site of injury. Plasma fibronectin (pFn) is secreted by the liver and circulates in the plasma. In the lung, cellular Fn (cFn) is secreted upon inflammation and injury. Both types of Fn are chemotactic factors for inflammatory cells and fibroblasts. Large numbers of inflammatory cells and fibroblasts infiltrate the lung during inflammatory episodes, which can lead to pulmonary fibrosis and ultimately death. Elevated levels of Fn have been detected in human clinical conditions such as neonatal RDS and BPD of the lung, and glomerular nephropathy of the kidney.

30

### The Role of UG

Amino acid analysis of purified human UG reveals that it is structurally similar but not identical to other "UG-like" proteins, e.g. rabbit UG. 39 of 70 amino acids are identical between human and rabbit UG (see Figure 1). The "UG-like" proteins, including human UG/CC10, rat CC10, mouse CC10, and rabbit UG, exhibit species-specific and tissue-specific antigenic differences, as well as differences in their tissue distribution and biochemical activities *in vitro*. UG-like proteins have been described in many different contexts with regard to tissue and species of origin, including rat lung, human urine, sputum, blood components, rabbit uterus, rat and human prostate, and human lung. At present there are no known physiological roles for these proteins.

Despite years of study, the biological roles of these proteins *in vivo* remain unclear. The absence of structural identity among UG-like proteins makes it impossible to predict whether a protein will possess *in vivo* therapeutic function in humans based on *in vitro* or other activity exhibited by a structurally related protein. For example, human uteroglobin binds less than 5% of the amount of progesterone as rabbit UG binds in the same assay. Human UG has a lower isoelectric point (4.6) than rabbit UG (5.4).

Stripp *et al.* (1996) have reported studies on a uteroglobin knockout mouse generated to eliminate expression of uteroglobin. The mouse has Clara cells which exhibit odd intracellular structures in place of uteroglobin secretion granules, but there is no other phenotype. This observation is highly significant because pulmonary function accompanied by pulmonary inflammation and fibrosis was expected. Moreover, this knockout mouse showed no evidence of renal, pancreatic, or reproductive abnormality, indicating that the uteroglobin protein had no significant role in controlling inflammation or fibrosis *in vivo*.

## OBJECTS OF THE INVENTION

Therefore, it is an object of the present invention to provide a pharmaceutical composition including a PLA<sub>2</sub> inhibiting effective amount of recombinant human uteroglobin (rhUG) or a fragment or derivative thereof and a pharmaceutically acceptable carrier or diluent.

It is a further object of the invention to provide a pharmaceutical composition including a fibronectin binding effective amount of recombinant human uteroglobin or a fragment or derivative thereof and a pharmaceutically acceptable carrier or diluent.

10 It is an additional object of the present invention to provide a pharmaceutical composition including a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of an active agent for treatment of a target indication.

Further, it is an object of the invention to provide a method for inhibiting PLA<sub>2</sub> enzymes *in vivo* in a mammal in need of such treatment, wherein the method includes administering to a mammal a PLA<sub>2</sub> inhibiting effective amount of native or recombinant human uteroglobin or a fragment or derivative thereof.

15 Still further, it is an object of the present invention to provide a method for treating or preventing an inflammatory condition in a patient in need of such treatment, wherein the method includes administering an anti-inflammatory effective amount of native or recombinant UG or a fragment or derivative thereof.

20 It is an object of the present invention to provide a method for treating or preventing a fibrotic condition in a patient in need of such treatment, wherein the method includes administering a fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

25 It is an additional object of the present invention to provide a method for treating or preventing an inflammatory or fibrotic condition characterized by a deficiency of endogenous UG, wherein the method includes administering a compensating amount of native or recombinant UG or a fragment or derivative thereof.

It is a further object of the present invention to provide a cosmetic composition comprising a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

5 Additionally, it is an object of the present invention to provide a blood supplement comprising a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

Finally, it is an object of the present invention to provide an assay quantitating uteroglobin-fibronectin complexes in a clinical sample.

## 10

## SUMMARY OF THE INVENTION

It has now been found that uteroglobin plays a central physiological role in inhibition of PLA<sub>2</sub>s and in prevention of fibronectin deposition and fibrosis *in vivo*. A combination of experiments performed in a new strain of transgenic uteroglobin "knockout" mice, and in a monkey model of neonatal respiratory distress syndrome  
15 (RDS) which involves pulmonary inflammation and fibrosis demonstrate these effects. The uteroglobin knockout mice of the present invention (hereinafter the "UG KO mice/mouse") exhibit lethal glomerular nephropathy and renal parenchymal fibrosis, as early and late onset diseases, respectively. Administration of exogenous Fn to normal mice causes Fn deposition in the kidneys, but administration of equimolar  
20 amounts of Fn and rhUG does not.

Reduction of PLA<sub>2</sub> activity *in vivo* has been demonstrated in the presence of rhUG. In a first experiment, the phenotype of the UG KO mice revealed that serum PLA<sub>2</sub> activity is significantly elevated in the absence of UG, compared to PLA<sub>2</sub> activity in littermates possessing a functional UG gene. In a second experiment,  
25 administration of rhUG to pre-term monkeys suffering from RDS was shown to inhibit PLA<sub>2</sub> activity in the extracellular fluids of the lungs.

Other experiments demonstrate that *in vitro* PLA<sub>2</sub> can degrade the artificial surfactant (typically Survanta) used in treatment of RDS and that UG can inhibit this degradation. These experiments demonstrate that UG mediates PLA<sub>2</sub> inhibition and  
30 Fn deposition *in vivo* following intratracheal or intravenous administration.

Experiments with the uteroglobin knockout mouse demonstrate that rhUG may be used to treat conditions in which uteroglobin is found to be deficient or the protein itself bears a loss-of-function mutation. It has now been discovered that rhUG may be used to treat or prevent inflammatory or fibrotic conditions in which functional  
5 endogenous uteroglobin is deficient in the circulation or at the site of inflammation or fibrosis. Reductions in the levels of hUG in serum and/or broncho-alveolar lavage fluids have been found in certain pulmonary inflammatory or fibrotic conditions, including pre-term infants at risk for developing neonatal BPD. It has been found that UG may be used to supplement deficient or defective endogenous uteroglobin to  
10 prevent or treat such inflammatory and fibrotic conditions.

According to one aspect, the invention provides a method of treating an inflammatory condition *in vivo* comprising administering to a patient in need of such treatment an anti-inflammatory effective amount of UG.

According to a further aspect, the invention provides a method of inhibiting  
15 soluble PLA<sub>2</sub> enzymes *in vivo*, which comprises administering to a patient in need of such treatment a PLA<sub>2</sub> inhibiting effective amount of UG.

In accordance with yet a further aspect, the invention provides a method for treating or preventing a fibrotic condition, which comprises administering to a patient in need of such treatment a fibronectin binding effective amount of UG.

20 A further aspect of the invention provides a method of treating or preventing fibrillogenesis by adding a fibronectin binding amount of UG.

In accordance with a further aspect, the invention provides a method for treating or preventing an inflammatory or fibrotic condition characterized by a deficiency of endogenous functional UG, which comprises administering to a patient  
25 in need of such treatment a compensating amount of UG.

The invention also provides pharmaceutical compositions comprising an effective amount of rhUG in association with a pharmaceutically acceptable carrier or diluent. The compositions may take the form of injectable solutions, and liquids or semi-aerosols for intratracheal administration.



According to a further aspect, the invention provides pharmaceutical compositions comprising UG and a lung surfactant (e.g. Survanta (a bovine lung extract from Abbott Labs) and Exosurf (a chemically synthetic lung surfactant from Glaxo-Wellcome)) in association with a pharmaceutically acceptable carrier or diluent.

5       The invention also includes pharmaceutical compositions containing a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of rhUG, an active agent for treatment of a target indication and a carrier. The PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of rhUG reduces inflammation and thereby insures that an effective amount of the active agent reaches the treatment site.

10       In another aspect, the invention provides an assay for quantitating uteroglobin-fibronectin complexes in a clinical sample, wherein a clinical sample suspected of containing a uteroglobin-fibronectin complex is contacted with an antigen capture agent, for example a monospecific rabbit polyclonal antibody, immobilized on an insoluble support; an antigen detection agent, for example an antibody specific for  
15       fibronectin, is added to the sample; and the presence of any complex bound to the support is detected using, for example, a secondary antibody, e.g., anti-IgG antibody conjugated to an enzyme such as horse radish peroxidase using a standard enzymatic reaction wherein the enzyme substrate is converted to a chromogenic or fluorogenic compound which is quantitated using standard spectrophotometric or fluorometric  
20       apparatus.

The invention also provides a cosmetic composition and a blood supplement including a PLA<sub>2</sub> inhibiting and/or fibronectin binding effective amount of human uteroglobin and a pharmaceutically acceptable carrier or diluent.

25

#### BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in more detail, with reference to the accompanying drawings, in which:

FIGURE 1 shows an alignment of UG-like proteins;

FIGURE 2A shows the intended targeting construct of the transgenic UG knockout mouse; the restriction sites are B - BamIII, E = EcoRI, H = HindIII; FIGURES 2B-2D show verification of the genetic construct in progeny of transgenic embryos by PCR and Southern blot analyses; (B) southern blot analyses of the targeted ES R1 cell clones, wherein Wt = wild type; (C) representative PCR analyses of genomic DNA from tail biopsies of offspring; the genotypes and their corresponding PCR products are as follows: UG<sup>+/+</sup>, 304 bp; UG<sup>+/+</sup>, 304 and 667 bp; UG<sup>-/-</sup>, 667 bp; (D) southern blot of mouse tail genomic DNA; FIGURE 2E shows confirmation of the absence of UG-mRNA in the lung tissues of UG<sup>-/-</sup> mice by RT-PCR analysis; RT-PCR analyses of total RNA extracted from the lung tissues of littermates with UG<sup>+/+</sup>, UG<sup>+/+</sup>, and UG<sup>-/-</sup> genotypes; a 273 bp RT-PCR product was detectable in the lungs of UG<sup>+/+</sup> and UG<sup>+/+</sup> but lacking from those of UG<sup>-/-</sup> mice; FIGURE 2F shows confirmation of the absence of UG protein in the lungs of UG<sup>-/-</sup> mice by Western analysis; proteins (30 micrograms each) from lung lysate were resolved by electrophoresis using 4-20% gradient SDS-Page under non-reducing conditions and immunoblotted using rabbit anti-mouse UG; FIGURE 2G shows confirmation of the absence of UG in lung tissue sections of the UG<sup>-/-</sup> mice using immunohistochemical methods in bronchiolar epithelial cells; the dark staining over the bronchiolar epithelial cells of UG<sup>+/+</sup> mouse (upper panel) indicates UG immunoreactivity; note the absence of immunoreactivity in UG<sup>-/-</sup> mouse lungs (lower panel).

FIGURES 3A-3J compare histopathological analyses of kidney sections from normal versus UG<sup>-/-</sup> mice, showing abnormal parenchymal fibrosis and glomerular Fn deposition in the knockout mice only; H & E staining of kidney sections from a UG<sup>+/+</sup> (A) and its UG<sup>-/-</sup> (B) littermate; (C) kidney section of a 10 month old mouse with severe parenchymal fibrosis; (D) a region of the same mouse kidney in (C) showing renal tubular hyperplasia (magnification 40X, g = glomerulus; f = fibrosis; t = tubule); (E) transmission electron microscopy of the glomerular deposit of a UG<sup>-/-</sup> mouse with severe renal disease (magnification 6000X); (F) the inset in (E) is magnified 60,000X, which shows the long striated fibrillar structures indicative of

collagen (col) and short diffuse ones consistent with Fn fibrils; (G) Fn immunofluorescence of a kidney section from a UG<sup>+/+</sup> mouse using murine Fn-antibody; (H) Fn-immunofluorescence of a kidney section from a UG<sup>-/-</sup> mouse with severe renal disease; Mason's trichrome staining of the kidney sections with UG<sup>+/+</sup> (I) and UG<sup>-/-</sup> (J) mice; the bluish staining over the glomeruli of UG<sup>-/-</sup> mouse kidney section is collagen (magnification approximately 40X).

FIGURE 4A shows the presence of Fn aggregates only in the kidneys of the UG<sup>-/-</sup> mice; immunoprecipitation and western blotting of Fn from plasma, kidney, and liver of UG<sup>+/+</sup> and UG<sup>-/-</sup> mice; a multimeric Fn band (bold arrow) was detected only in the kidney lysates of UG<sup>-/-</sup> mice.

FIGURES 4B and 4C show the formation of UG-Fn complexes *in vitro*; (B) equimolar concentrations of UG and Fn were incubated, immunoprecipitated with and detected by Western blotting with either Fn or UG antibody; the immunoprecipitates contain both Fn (lane 2, upper panel) and UG (lane 2, lower panel); lanes 1 of both panels represent Fn and UG standards; (C) equimolar concentrations of <sup>125</sup>I-UG and Fn were incubated at 4°C for 1 hour and the resulting complex was resolved by electrophoresis on 6% non-reducing, non-denaturing polyacrylamide gels; lane 1, coomassie blue stained Fn-UG heteromer; lane 2, its autoradiogram.

FIGURE 4D shows the presence of UG-Fn complexes in the plasma of normal but not UG<sup>-/-</sup> mice; immunoprecipitation of plasma from UG<sup>+/+</sup> and UG<sup>-/-</sup> mice with Fn-antibody and western blotting with Fn and UG antibodies; Fn (upper panel); UG (lower panel); std = standards for UG and Fn.

FIGURE 4E shows the dose-dependent inhibition of Fn self-aggregation by UG *in vitro*; affinity-crosslinking of <sup>125</sup>I-Fn with unlabeled Fn in the absence (lane 2) and presence of varying amounts of UG (lanes 3-5); the intensity of the very high molecular weight, radioactive Fn band (lane 2) formed in the absence of UG is reduced in a dose-dependent manner; lane 1, <sup>125</sup>I-Fn with unlabeled Fn in the absence of UG and DSS; open arrowhead - multimeric Fn; lower thin arrow = 220 kDa Fn.

FIGURE 4F shows the inhibition of Fn-collagen complex formation by UG; affinity crosslinking of  $^{125}\text{I}$ -collagen I with unlabeled Fn in the absence of (lane 3) and presence (lane 4) of UG; lane 1, coomassie blue-stained collagen I; alpha<sub>1</sub>-alpha<sub>1</sub> chain of collagen I and alpha<sub>2</sub>-alpha<sub>2</sub> chain of collagen I; lane 2,  $^{125}\text{I}$ -collagen I and unlabeled Fn in the absence of UG and DSS.

FIGURES 5A-5F show the immunohistochemical analysis of Fn deposition in the kidneys of normal and UG<sup>-/-</sup> mice only in the absence of UG; (A) kidney section of a wild-type mouse that received a mixture of equimolar concentrations of Fn and UG intravenously; (B) UG<sup>+/+</sup> mouse that received the same dose of Fn as in (A) but without UG; (C) apparently healthy, UG<sup>-/-</sup> mouse receiving a mixture of Fn and UG; (D) UG<sup>-/-</sup> mouse receiving Fn alone (same dose as in (C)), but without UG; (E) Fn-fibrillogenesis by cultured cells grown in medium supplemented with soluble hFn alone; (F) a cell culture identical to one in (E) which was fed with medium containing a mixture of equimolar concentrations of soluble hFn and UG (magnification 40X, g = glomerulus).

FIGURES 6A-6B show the format for a diagnostic assay to detect UG-Fn complexes in clinical samples.

FIGURE 7 shows the passage of UG dimer through an 8.0 kDa MWCO dialysis membrane but not a 3.5 kDa MWCO dialysis membrane.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

### rhUG

The rhUG of the invention has substantially the same amino acid sequence as that of the native human UG protein. An amino acid sequence having "substantially the same" amino acid sequence as that of the native human protein includes rhUG having at least 75% identity to the native human protein. In a preferred embodiment, rhUG has at least 85% identity, and in a most preferred embodiment, rhUG has at least 98% identity to the native UG.

Also included in the method of the present invention is the use of fragments or derivatives of UG. A "fragment" of UG refers to a portion of the native hUG

amino acid sequence having six or more contiguous amino acids of the native protein sequence. The term "derivative" refers to peptide analogs of UG, including one or more amino acid substitutions and/or the addition of one or more chemical moieties, e.g., acylating agents or sulfonating agents, with the proviso that the derivative  
 5 retains the biological activity of the parent molecule.

Further, the UG used in the method of the present invention is substantially pure. The term "substantially pure" refers to UG having a purity of about 75% to about 100%. In a preferred embodiment, UG has a purity of about 90% to about 100%, and in the most preferred embodiment, UG has a purity of at least 95%.

#### 10 Clinical Uses of UG

The invention provides, in another aspect, a method of treating or preventing an inflammatory or fibrotic condition comprising administering to a mammal, which may be animal or human, an effective amount of UG.

The following non-limiting list of conditions are representative examples of  
 15 those associated with UG deficiencies, excessive PLA<sub>2</sub> activity, and fibronectin deposition.

**Table 2. Clinical Uses of Recombinant Uteroglobulin  
(Grouped by UG Property)**

20	<u>UG Property</u>	<u>Condition(s)</u>
	UG deficiency	(1) Neonatal broncho-pulmonary dysplasia; (2) Complications of hemodialysis; (3) Bleomycin lung; (4) Chronic obstructive pulmonary disease; (5) Emphysema
	Excessive PLA <sub>2</sub> activity (inflammation)	(1) Systemic inflammation; (2) Asthma; (3) Cystic fibrosis; (4) Ocular inflammation, including Autoimmune uveitis and corneal transplant surgery; (5) Conditions associated with obstetrics and gynecology, including premature labor and fertility (ex vivo)
25	Excessive PLA <sub>2</sub> activity (Immune modulation)	(1) Autoimmune diseases, including rheumatoid arthritis, Type I diabetes, Inflammatory bowel disease, and Crohn's disease; (2) Transplanted organ rejection
	Fibronectin deposition	(1) Renal fibroses (2) Pulmonary fibroses, including idiopathic pulmonary fibrosis; (3) Vascular fibrosis

Typical relationships between UG deficiency, PLA<sub>2</sub> activity and fibronectin aggregation and deposition in human inflammatory/fibrotic conditions are summarized below.

5

#### **Neonatal Broncho-Pulmonary Dysplasia (Neonatal BPD)**

Neonatal BPD is characterized by severe inflammation and irreversible fibrosis of lung tissue in newborn infants, usually as a result of respiratory distress syndrome (RDS). However, this condition may also be caused by meconium aspiration syndrome or infection.

10

A deficiency of hUG has been implicated in this condition because the synthesis of pulmonary hUG may be coregulated with surfactant, which starts late in gestation. Thus, severely premature neonates may lack UG as well as surfactant. hUG deficiency may cause increased PLA<sub>2</sub> activity and Fn-related fibrosis, which are associated with the inflammation and fibrosis seen in neonatal BPD. Some infants do not respond to synthetic surfactant, which may be due to excess PLA<sub>2</sub> activity. Thus, UG may be used to treat neonatal BPD.

15

The preferred route of administration is direct instillation via the endotracheal or the systemic routes.

20

#### **Multiple Organ Failure (MOF)**

Excessive PLA<sub>2</sub> activity has been implicated in MOF due to bacterial sepsis or trauma. This condition is characterized by a systemic inflammatory response, involving rapid, massive tissue damage and loss of organ function in the lungs, kidney, pancreas, intestines, and vasculature. Recent evidence points to the MOF trigger as elevated systemic soluble phospholipase A<sub>2</sub> activity, its direct lysis of tissue cell membranes, and hydrolysis of essential phospholipids, such as lung surfactants. Attempts to inhibit PLA<sub>2</sub> directly in clinical settings have been unsuccessful.

25

In MOF, the amount of endogenous UG is insufficient to counter the super-activation of PLA<sub>2</sub>. Exogenously supplied UG can be used to combat MOF.

30

Remote organ failure (ROF) involves damage to organs other than the organ primarily affected by trauma or infection. Often remote organ failure involves more than one remote organ, resulting in multiple organ failure. For example, pancreatitis is an inflammation of the pancreas in response to alcohol intake, infection, or trauma, that may result in adult respiratory distress syndrome (ARDS), acute renal failure (ARF), and systemic shock. An episode of inflammatory bowel disease or peritonitis can result in ROF/MOF. ROF/MOF is associated with high levels of circulating, activated PLA<sub>2</sub>. The systemic application of hUG could prevent ROF/MOF. The immediate injection of UG in patients with ROF/MOF, could reduce the severity or eliminate the PLA<sub>2</sub> mediated organ failure and shock.

#### Pancreatitis

All forms of pancreatitis involve elevated Type I soluble PLA<sub>2</sub> activity, both systemic and local. Pancreatitis often results in pulmonary insufficiency or ARDS, characterized by elevated soluble PLA<sub>2</sub> activity in the lungs. Therefore, as an inhibitor of soluble Type I PLA<sub>2</sub>s *in vivo*, UG is an excellent candidate for treatment of two forms of acute pancreatitis, and as a preventative measure of pulmonary insufficiency in all acute forms of pancreatitis.

The preferred route of administration is by the intravenous route.

#### Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD), including ulcerative colitis, diverticulitis, and Crohn's disease, is characterized by elevated local production and activity of Type II soluble PLA<sub>2</sub>. Circulating soluble PLA<sub>2</sub> activity may also be elevated in IBD. IBD causes pulmonary insufficiency or ARDS in severe cases, as a result of elevated PLA<sub>2</sub> activity (which is similar to pancreatitis).

The rationale for the application of exogenous UG in IBD is identical to that of pancreatitis: to downregulate the inflammatory response by inhibiting PLA<sub>2</sub>, F<sub>n</sub> aggregation and/or deposition and to prevent remote organ involvement (lungs and kidneys).

The preferred route of administration is by the intravenous route in hospitalized patients.

### **Bacterial Pneumonia**

- 5        BAL fluids of patients who have survived bacterial pneumonia were shown to have 2-3X higher levels of UG than those who died. Bacterial infection of the lungs may overactivate the endogenous soluble PLA<sub>2</sub>. UG may be administered to inhibit or control this effect.

- 10       The preferred route of administration is via the intratracheal route if the patient is intubated or intravenous if not.

### **Complications of Dialysis**

- 15       The major complication of dialysis is thromboses, i.e., spontaneously formed blood clots. These often plug the vascular access port, impairing treatment, as well as causing ischemic, sometimes life-threatening episodes, in the patient. A second problem with hemodialysis patients is inflammation and/or fibrosis of the proximal vein which returns the dialyzed blood to the patient's main circulation. Fibrosis of the proximal vein is usually detected as an increase in resistance, or pressure, against the return of the dialyzed blood. A third problem is fibrosis and closure of the vascular access site, or fistula. A fourth problem is accelerated atherosclerosis and a fifth is loss of residual renal function, most likely due to Fn deposition.

- 20       The possibility that endogenous UG is dialyzed away during the procedure provides an explanation for these problems. The selective removal of endogenous UG leaves circulating Fn free to aggregate, forming the foci for blood clot formation or to deposit on red blood cells, priming them for a clotting response by sticking to each other or to the vascular lumen. Transglutaminases (TGs) are enzymes responsible for building macromolecular lattices found in basement membranes, skin and blood clots. In the absence of free UG competing as a substrate for activated TGs, Fn and other components of blood clots are crosslinked.



Inflammation and fibrosis of both the proximal vein and the vascular access site, as well as accelerated atherosclerosis, may be explained by the deposition of Fn in the vascular lumen. Fibronectin deposition on the vascular endothelia promotes platelet and white blood cell adherence, both of which may be aggravated in the  
5 absence of PLA<sub>2</sub> inhibition. Vascular deposits of Fn may also promote local deposits of fat, cholesterol and protein found in atherosclerotic plaque. Fibronectin is known to be a major component of atherosclerotic plaque, as well as renal glomerular deposits associated with nephropathy and loss of primary and residual renal function. Therefore, UG administration may reduce or eliminate these problems by reducing  
10 inflammation and fibronectin deposition.

The preferred route of administration of UG would be intravenous infusion before, during or after dialysis.

Alternatively, the loss of endogenous UG may be prevented by addition of UG to the dialysis buffer or precoating the dialysis membrane with UG or both.

15

### Organ Transplants

The term "organ" refers, for example, to solid organs, such as kidney, liver and heart, as well as bone marrow, cornea and skin.

There are two types of organ transplant rejection: acute and chronic. Acute  
20 rejection is an inflammatory process involving PLA<sub>2</sub> activity and infiltration by inflammatory cells that often destroys the graft.

Chronic rejection involves Fn-mediated fibrosis of the graft, including atherosclerosis confined to the graft. Thus, administration of UG may be used to treat or prevent both acute and chronic graft rejection.

25 The preferred route of administration is by injection.

Another aspect of organ transplantation is ischemia of the organ before removal from the donor, during transport and in the recipient, which contributes to acute rejection. Ischemia is known to result in elevated PLA<sub>2</sub> activity and tissue necrosis. Hence, UG could be used to prevent such ischemia. The preferred form

of UG is as a perfusion liquid or storage buffer in which the ex vivo organ is preserved.

### **Prevention of Type I Diabetes**

5

Type 1 diabetes arises from the destruction of pancreatic tissue by an autoimmune response. The pancreas normally secretes soluble PLA<sub>2</sub>s and hUG into the circulation. Necrotic lesions have been reported in the pancreas of the uteroglobin knockout (KO) mouse of the present invention (herein referred to as the "UG KO mouse").

10

In the absence of uteroglobin, the UG KO mouse exhibits similar pancreatic tissue destruction which could trigger an autoimmune response. Thus UG may be used to prevent or halt the slow progression of Type 1 diabetes. The preferred route of administration is by injection.

15

### **Prevention and Treatment of Nephropathy**

Renal Fn deposits and fibrosis in the UG KO mouse are similar to Fn deposits and fibrosis in human nephropathies. Thus, UG administration may prevent or slow the progression of nephropathy in patients at risk, such as Type II diabetes.

20

### **Prevention and Treatment of Ocular Inflammation**

Ocular inflammation, including uveitis, retinitis, and inflammation following surgery, is characterized by increased PLA<sub>2</sub> activity. Therefore, UG may be administered topically, intraocularly, or systemically to reduce ocular inflammation.

25

### **Arteriosclerosis**

Arteriosclerosis is a fibrotic thickening of blood vessels throughout the body. It is initiated and/or mediated by Fn deposition on the walls of the vasculature. Atherosclerosis is a form of arteriosclerosis involving cholesterol deposition, in addition to Fn deposition. Therefore, UG may be administered to prevent or reduce arteriosclerosis.

30

**Acute Renal Failure**

Acute renal failure (ARF) is typically a consequence of remote organ inflammation, infection or direct trauma, which results in release and activation of soluble PLA<sub>2</sub> in the circulation. Damage to the kidneys during ARF can be quite severe, with acute tissue damage promoted by inflammation and may resolve into fibrosis of the kidney, leading to reduced kidney function in the long term. The anti-inflammatory and anti-fibrotic properties of UG are particularly relevant in the kidney as shown by the UG KO mouse.

The preferred route of administration is by injection or systemic administration.

Overall, the following non-limiting list of conditions are those associated with inhibition of PLA<sub>2</sub> and/or fibronectin deposition, each of which are candidates for treatment or prevention by the method of present invention:

15

- |                    |   |
|--------------------|---|
| Joint/Bone:        | rheumatoid arthritis;   |
| Autoimmune:        | rheumatoid arthritis, multiple sclerosis, Type 1 diabetes, uveitis, psoriasis, systemic lupus erythematosus (SLE), and Crohn's disease;   |
| 20 Pancreas:       | pancreatitis;   |
| Peritoneum:        | peritonitis, appendicitis;  |
| Vascular/systemic: | septic shock; collagen vascular disease, arteriosclerosis, atherosclerosis, anaphylactic shock, schistosomiasis, and trauma-induced shock;  |
| 25 Renal:          | acute renal failure, bacterial infection of the kidneys, inflammation due to renal tumors, prevention of fibrosis resulting from chemotherapy or antibody therapy, prevention of diabetic nephropathy, prevention and/or treatment of idiopathic nephropathy; |
| 30 Liver:          | hepatitis, viral hepatitis, and cirrhosis;  |

	<b>Bladder:</b>	cystitis, inflammation of the urethra, inflammation of the ureter, and bladder inflammation such as interstitial cystitis;
5	<b>Reproductive/female:</b>	vaginitis, inflamed cervix, pelvic inflammatory disease, inflammation of the ovary (salpingitis), endometriosis, vaginal candidiasis, and inflammation or fibrosis of the fallopian tubes;
10	<b>Reproductive/male:</b>	penile inflammation, prostate inflammation, inflammation of seminal tubules and vesicles, testicular inflammation, and inflammation of vas deferens, epididymis, and prostate gland;
15	<b>Ocular:</b>	uveitis, retinitis, trauma, burn damage due to chemical or smoke, ocular inflammation due to CMV retinitis, conjunctivitis (bacterial infection), viral infection, ocular inflammation due to infectious agent, ocular inflammation following ocular surgery, including cataract removal, laser surgery, corneal transplant, tumor removal, ocular inflammation due to retinoblastoma (tumor), ocular inflammation due to radiation exposure, inflammation due to allergic response;
20		
	<b>Heart:</b>	Endocarditis
25	<b>Lungs:</b>	bronchial asthma, ARDS, pneumonia, idiopathic pulmonary fibrosis, pulmonary fibrosis resulting from chemotherapy (bleomycin, methotrexate), pulmonary fibrosis resulting from exposure to environmental chemicals (asbestos, cleaning fluids, pollutants, e.g. dioxin and PCB's in automobile exhaust), smoke inhalation, pulmonary inflammation during recovery from drowning, and neonatal RDS;
30		

- Gut:** inflammatory bowel disease, colitis, Crohn's disease, direticulitis, neonatal necrotizing enterocolitis, inflammation due to an infectious agent, rotavirus, polio virus, HIV, stomach ulcers, gastro-esophageal reflux disease, and tonsillitis;
- Hemorrhoids;**
- Transplants:** administration following transplant surgery for any organ or tissue to control inflammation or fibrosis and rejections;
- Ears:** Otitis media; and
- Skin:** psoriasis, hives, allergic and dermatitis, scleroderma, contact dermatitis, chemical dermatitis (due to poison ivy, poison oak, and exposure to chemicals like PCB's, chlorine, ammonia, (cleaning agents, toxic agents).

Moreover, UG may be administered either alone or in combination with other active agents or compositions typically used in the treatment or prevention of the above-identified disease conditions. Such active agents or compositions include, but are not limited to steroids, non-steroidal anti-inflammatories (NSAIDs), chemotherapeutics, analgesics, immunotherapeutics, antiviral agents, antifungal agents, vaccines, immunosuppressants, hematopoietic growth factors, hormones, cytokines, antibodies, antithrombotics, cardiovascular drugs, or fertility drugs. Also included are oral tolerance drugs, vitamins and minerals.

Moreover, UG may be administered as a carrier for the suppression of inflammation at the site of injection or administration of another therapeutic or prophylactic agent to suppress inflammation or an immune response caused by such agent. In this regard, UG would allow for administration of an effective amount of the active agent to the target indication.

UG may also be administered as a cosmetic composition for use in limiting fibrosis, scar or keloid formation resulting from wound healing, or during a dermal inflammatory response.

Further, UG may be used as a blood supplement, i.e., as an additive to  
5 synthetic or donated human blood.

Finally, UG may be used to enhance the rate of artificial insemination by adding to sperm, in vitro fertilized eggs, and embryos prior to transfer to the maternal uterus in humans and other mammals.

The present invention relates to the use of UG in the prevention or treatment  
10 of PLA<sub>2</sub> and fibronectin associated conditions. With regard to prevention of a disease condition, "prevention" refers to preventing the development of disease in a susceptible or potentially susceptible population, or limiting its severity or progression, whereas the term "treatment" refers to the amelioration of a disease or pathological condition.

15 UG may be administered intravenously or, in the case of treatment of neonatal RDS/BPD and adult RDS, in the form of a liquid or semi-aerosol via the intratracheal tube. Other viable routes of administration include topical, ocular, dermal, transdermal, anal, systemic, intramuscular, slow release, oral, vaginal, intraduodenal, intraperitoneal, and intracolonic. Such compositions can be administered to a subject  
20 or patient in need of such administration in dosages and by techniques well known to those skilled in the medical, nutritional or veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular subject or patient, and the route of administration. The compositions of the present invention may also be administered in a controlled-release formulation. The compositions can be co-  
25 administered or sequentially administered with other active agents, again, taking into consideration such factors as the age, sex, weight, and condition of the particular subject or patient, and, the route of administration.

Examples of compositions of the invention include edible compositions for oral administration such as solid or liquid formulations, for instance, capsules, tablets,  
30 pills, and the like liquid preparations for orifice, e.g., oral, nasal, anal, vaginal etc.,

formulation such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration), such as sterile suspensions or emulsions. However, the active ingredient in the compositions may complex with proteins such that when administered into the bloodstream, clotting may occur due to precipitation of blood proteins; and, the skilled artisan should take this into account.

In such compositions UG may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, DMSO, ethanol, or the like. UG can be provided in lyophilized form for reconstituting, for instance, isotonic aqueous, saline, glucose, or DMSO buffer. In certain saline solutions, some precipitation of rhUG has been observed; and this observation may be employed as a means to isolate inventive compounds, e.g., by a "salting out" procedure.

Further, the invention also comprehends a kit wherein UG is provided. The kit can include a separate container containing a suitable carrier, diluent or excipient.

15 The kit can include an additional agent which reduces or alleviates the ill effects of the above-identified conditions for co- or sequential-administration. The additional agent(s) can be provided in separate container(s) or in admixture with UG. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration.

20 The invention also contemplates a method for treating or preventing an inflammatory or fibrotic condition characterized by a deficiency of endogenous functional UG, which comprises administering to a patient in need of such treatment a compensating amount of UG. The term "compensating amount" means an amount of UG required to bring the local pulmonary or systemic concentration of total UG (endogenous functional UG and exogenous UG) to within its normal range. More specifically, the normal range for local pulmonary concentration of endogenous UG is about > 50 micrograms UG/milligram albumin or > 50 micrograms/liter. The normal range for serum UG concentration is > 15 micrograms/liter.

The compositions of the invention comprise native and/or recombinant hUG in an amount effective to achieve the intended purpose, namely increased plasma or

tissue levels of UG to produce the desired effect of selective inhibition of PLA<sub>2</sub>s and reduced inflammation and/or binding of fibronectin to mitigate its aggregation and/or deposition in fibrotic conditions. The compositions comprise an effective amount of substantially pure native and/or recombinant human UG, in association with a  
5 pharmaceutically acceptable carrier or diluent.

In a further aspect, the compositions of the invention comprise an effective amount of native and/or recombinant hUG and lung surfactant, in association with a pharmaceutically acceptable carrier or diluent. Local intratracheal administration of UG to the lungs, sufficient for inhibition of PLA<sub>2</sub> activity and/or fibronectin  
10 deposition requires quantities of substantially pure UG in the range of 0.2 µg/kg to 500 mg/kg of protein in single or multiple dosages. The UG is usually administered in an amount of a single bolus of 20 ng/kg to 500 mg/kg, in single or multiple doses, or as a continuous infusion of up to 10 grams.

Native and/or recombinant hUG may also be administered in conjunction with  
15 artificial lung surfactant, such as Surfacta, via the intratracheal route. UG and Surfacta (5 mls/kg) are co-administered and UG does not bind surfactant, preventing it from functioning therapeutically. The lung surfactant is generally present in the composition an amount of about 10-90 % by weight, more usually about 20-80 % by weight. The surfactant, by virtue of its very low surface tension spreads out over the  
20 internal surface of the lungs, carrying the UG with it. Systemic administration of UG via intravenous injection, sufficient for inhibition of PLA<sub>2</sub> activity and/or fibronectin deposition, requires quantities of substantially pure native and/or recombinant hUG in the range of 0.5 µg to a continuous infusion of several grams of protein over an extended period of time (days).

25 Suitable formulations for injection and semi-aerosol intratracheal delivery include aqueous solutions of native and/or recombinant hUG optionally with viscosity modifiers and stabilizers.

The term "PLA<sub>2</sub> inhibiting effective amount" as used herein means the amount of UG which inhibits PLA<sub>2</sub> activity and which reduces or alleviates inflammation in  
30 the tissue or body of the patient. The term "fibronectin binding effective amount"



means that amount of UG which binds fibronectin to reduce aggregation and/or deposition thereof, and prevent or reduce fibrillogenesis or fibrosis. The term "anti-inflammatory amount" as used herein means the amount which reduces or alleviates inflammation in the tissue or body. Typically, the amount of UG administered to adults for the treatment of inflammatory and fibrotic conditions will be single boluses of 0.2  $\mu$ g/kg to 500 mg/kg or up to several grams administered over an extended period of time. For neonates, in the treatment of neonatal RDS, the range will typically be 50 nanograms/kg to 100 mg/kg in single boluses or up to 10 grams administered continuously over an extended period of time. Effective and safe rates of continuous infusion are between 50 ng/kg/hour to 500 mg/kg/hour.

### EXAMPLES

The invention will now be further described with reference to the following non-limiting examples. Parts and percentages are by weight unless otherwise stated.

#### Example 1: In Vivo Experiments

Recombinant human UG was obtained by the method of Mantile *et al.* (1993).

One male and one female of the species *P. cyanocephalus*, weighing approximately 400 grams each were delivered by C-section at 142 days of gestation. This is an established model of RDS (Coalson, J.J., et al. Baboon Model of BPD. II: Pathologic features. Exp. Mol. Pathol. 37: 355-350 (1982)).

After delivery, the infants were anesthetized with ketamine (10 mg/kg) and intubated with a 2.5 mm diameter endotracheal tube. Blood gases and pressure were monitored via an arterial line placed by percutaneous injection into the radial artery. A deep venous line was placed percutaneously into the saphenous vein through which fluids, antibiotics, and drugs were administered. Animals were maintained on servo-controlled infrared warmers and ventilated with a standard time-cycled, pressure-regulated ventilator with humidifiers maintained at 36-37°C. Initial setting were  $\text{FiO}_2$  1.0, rate 40/min., I/E ratio 1:1.5, positive end expiratory pressure (PHEP) at 4

cm H<sub>2</sub>O, and peak inspiratory pressure (PIP) as required for adequate chest excursion. FiO<sub>2</sub> was kept at 1.0 and PIP was regulated to maintain PaCO<sub>2</sub> at 40±10 torr. Blood gases, hematocrit, electrolytes, prothrombin time, partial thromboplastin time and dextrostix were monitored hourly. Blood drawn for studies was replaced  
5 volumetrically with heparinized adult baboon blood. Intravenous fluids were administered with electrolytes at 10 cc/kg/hr and were increased as needed when heart rate exceeded 180 beats/min. Sodium bicarbonate (2 meq/kg) was administered when the base deficit exceeded -10. Ampicillin (50 mg/kg/day in two divided doses) and Gentamicin (5 mg/kg/day in two divided doses) was given continuously for the  
10 duration of the experiment.

One animal received surfactant plus PBS (treatment no. 1), and the second animal (treatment no. 2) received surfactant plus two doses of 1 mg/kg of rhUG. Both surfactant and rhUG were administered directly to the lungs through the endotracheal tube. The surfactant used was Survanta (Ross Labs), a surfactant  
15 preparation derived from bovine lung tissue, containing surfactant apoproteins B and C in addition to phospholipids. The first dose of rhUG was given with the surfactant and the second administered four hours after the first. The animals were monitored for arterial blood gases, electrolytes and EKG. They were sacrificed 50 hours after the initiation of surfactant therapy. The lungs were lavaged at 24 and 48 hours with  
20 PBS containing protease inhibitors (PMSF, 10 µg/ml leupeptin, 10 µg/ml of pepstatin and bacitracin). They were frozen at -80°C until assayed for PLA<sub>2</sub> activity. Total proteins were determined by Bradford method (BioRad). The PLA<sub>2</sub> activity in the lung lavages were measured according to Levin et al. (1986; *supra*) and are presented in the following Table.

Table 3. Results of In Vivo Testing of UG

5	Treatment #	Time	Lung lavage PLA <sub>2</sub> activity (ccpm/10 µg protein)
	1	24 hr 48 hr	3030 2607
	2	24 hr 48 hr	1739 996

The data given above are the mean of two determinations. The results show that endotracheal administration of rhUG inhibits PLA<sub>2</sub> *in vivo*. The animals which received surfactant and rhUG had an appreciably lower PLA<sub>2</sub> activity in their lung lavage fluid compared with the animals that received surfactant without rhUG. The data confirm that administration of rhUG in conjunction with surfactant is beneficial in protecting surfactant phospholipids.

15

**Example 2: Inhibition of Hydrolysis of Artificial  
Surfactant by Soluble PLA<sub>2</sub> *in vitro***

RhUG inhibits hydrolysis of artificial surfactant by soluble PLA<sub>2</sub>s *in vitro*. Survanta is an artificial surfactant derived from bovine lung and is used to treat pre-term neonates with RDS and adults with RDS (ARDS). Hydrolysis of Survanta by a Group I soluble PLA<sub>2</sub>, i.e. porcine, pancreatic PLA<sub>2</sub> (Boehringer Mannheim) is characterized by its ability to compete as a substrate with a fluorescent phosphatidylcholine substrate (Cayman Chemicals), generating arachidonic acid as a product.

25

Survanta is a substrate for *in vitro* degradation by Group I soluble PLA<sub>2</sub>s. Survanta is rapidly degraded *in vitro* by PLA<sub>2</sub>s found in the extracellular fluids of a human lung. RhUG inhibits degradation of Survanta *in vitro*.

30

**Example 3: Construction of UG knockout mouse**

A transgenic UG KO mouse was created for the purpose of determining the role of uteroglobin in mammalian physiology, as well as to generate a model for UG as a therapeutic in several inflammatory clinical conditions. The first step was to construct an appropriate DNA vector with which to target and interrupt the endogenous murine uteroglobin gene. The 3.2 kb BamHI-EcoRI DNA fragment containing exon 3 and flanking sequences of the uteroglobin gene from the 129/SVJ mouse strain (Ray, 1993) were subcloned into the corresponding sites of the pPNW vector as described in Lei et al (1996). A 0.9 kb fragment containing part of exon 2 and its upstream sequence was amplified by PCR (with primers Primer-L (from Intron 1): 5'-TTC CAA GGC AGA ACA TTT GAG AC-3'; Primer-R (from Exon 2): 5'-TCT GAG CCA GGG TTG AAA GG C-3') with NotI and XhoI restriction sites engineered into the termini for directional subcloning into the gene targeting vector. In this construct, 79 bp of Exon 2 encoding 27 amino acids were deleted. The PCR fragment was placed upstream of the gene encoding neomycin resistance in pPNW, generating the gene targeting vector, pPNWUG. The vector is shown in Figure 2A, in which the PGK-neo cassette interrupts the uteroglobin gene, disrupting the protein coding sequence.

The pPNWUG gene targeting vector was linearized with NotI and electroporated into ES R<sub>1</sub> cells according to Nagy, A., et al. PNAS 90:8424 (1993). Gancyclovir and G-418 selection of the electroporated cells yielded 156 clones. Southern (DNA) blot analysis identified a 5.1 kb HindIII fragment of the wild-type uteroglobin allele and an additional 8.2 kb HindIII fragment resulting from homologous recombination in three out of the 156 clones, shown in Figure 2B. These ES R<sub>1</sub> clones were injected into C57BL/6 blastocysts according to Capecchi, Science 244: 1288 (1989). Two different lines of mice, descended from different chimeric founders, were generated. Heterozygous offspring (UG<sup>+/+</sup>) carrying the targeted uteroglobin gene locus were mated and the genotypes of the progeny were analyzed by PCR shown in Figure 2C, as well as Southern blot, shown in Figure 2D.

**Example 4: Verification of UG gene knockout and absence of murine UG (mUG) protein**

In order to verify that the homozygous knockout mice ( $UG^{-/-}$ ) did not possess  
5 any detectable mUG, the uteroglobin gene-targeted mice were tested for expression  
ofm UG-mRNA and mUG protein in several organs including the lungs. An  
experimental protocol was approved by the institutional animal care and use  
committee. Total RNAs were isolated from different organs of  $UG^{+/+}$ ,  $UG^{+/-}$ , and  
 $UG^{-/-}$  mice. The reverse transcribed-polymerase chain reaction (RT-PCR) was used  
10 to detect mUG-mRNA. Target molecules were reverse transcribed using a mUG-  
specific primer, mPr (5'- ATC TTG CTT ACA CAG AGG ACT TG-3'), and the  
cDNA generated was amplified using PCR primers mPr and mPl (5'-ATC GCC ATC  
ACA ATC ACT GT-3'). The PCR product was hybridized with an oligonucleotide  
probe, mPp (5'-ATC AGA GTC TGG TTA TGT GGC ATC C-3') derived from  
15 exon-2 of the UG gene sequence. The primers and the probe used in mouse GAPDH  
RT-PCR are as follows: mGAPDH-r (5'-GGC ATC GAA GGT GGA AGA GT-3');  
mGAPDH-l (5'-ATG GCC TTC CGT GTT CCT AC-3'); mGAPDH-p (5'-GAA GGT  
GGT GAA GCA GGC ATC TGA GG-3'). Figure 2E shows that mUG-mRNA was  
detected in the lungs of  $UG^{+/+}$ , and  $UG^{+/-}$ , but not  $UG^{-/-}$  mice. Similar data (not  
20 shown) show that mUG-mRNA is not present in either the prostate or uteri of  $UG^{-/-}$   
mice, but is present in the mice with an intact uteroglobin gene.

Immunoprecipitation and Western blot analyses of mUG protein in the lungs  
yielded similar corroborative results, shown in Figure 2F. Tissue lysates from the  
kidneys, liver, and the lungs of the  $UG^{+/+}$  and  $UG^{-/-}$  mice were prepared by  
25 homogenizing in a buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.2%  
deoxycholate, 150 mM NaCl, 5 mM EDTA) containing 2 mM phenylmethylsulfonyl  
fluoride and 20  $\mu$ g/mL each of aprotinin, leupeptin, and pepstatin A. The  
homogenates were centrifuged at 17,500 x g for 30 min at 4°C and  
immunoprecipitated as described (E. Harlow and D. Lane, Antibodies; a laboratory  
30 manual, 1st Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY,  
1988) by incubating tissue lysates or plasma proteins (1 mg/mL) with rabbit antibody

against murine Fn (1:100 dilution). Co-immunoprecipitation of purified murine Fn (mFn) and rhUG (Mantile, G, et al., J. Biol. Chem. 267: 20343 (1993)) was performed by incubating equimolar concentrations of mFn with rhUG in the presence of 10% glycerol, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 4.3 mM sodium phosphate at 4°C for 1 hr., followed by adding anti-mFn antibody (1:100 dilution). Equal amounts of extracted tissue proteins (30 µg) or immunoprecipitates were resolved either on 4-20% or 6% SDS-polyacrylamide gels under reducing conditions, followed by Western blotting with rabbit antibodies against either murine Fn (1:2000 dilution) or UG (1:2000 dilution). No mUG was detected in tissues or fluids from the UG<sup>-/-</sup> mice, while tissues from UG<sup>+/+</sup> and UG<sup>+/-</sup> mice did contain the mUG protein.

Finally, histopathological analyses of the lungs of UG<sup>-/-</sup>, only, lacked mUG-specific immunostaining in bronchiolar epithelial cells. Lung tissues from UG<sup>-/-</sup>, UG<sup>+/-</sup> and UG<sup>+/+</sup> mice were fixed in Bouin's fluid or in 10% neutral buffered formalin fixatives, embedded in paraffin and sectioned at 4-6 microns. They were stained with hemotoxylin and eosin (H & E). Selected tissues were stained by Masson's trichrome method for collagen detection, PTAH for fibrin, or Congo Red for amyloid protein. For immunohistochemical detection of mUG and mFn, the Vectastain rabbit Elite ABC kit (Vector Laboratories) was used. The rabbit antibody (CytImmune) to mUG was raised by using a synthetic peptide (Peptide Technologies, Inc.) corresponding to mUG amino acid sequence (Lys28 to Thr49, specifically KPFNPGSDLQNAGTQLKRLVDT). The rabbit antibody to mFn (GIBCO BRL) was used at a dilution of 1:1000, and the antibody to mUG was used at 1:500.

These three sets of results confirm that the homozygous uteroglobin knockout mouse, UG<sup>-/-</sup>, lacks mUG protein, or any detectable piece of the protein.

#### **Example 5: Phenotype of uteroglobin knockout mouse**

Of the 179 mice born to crosses of UG<sup>+/+</sup> mice, 46 (26%) were of the +/+, 90 (50%) of the +/- and 43 (24%) of the UG<sup>-/-</sup> genotype, indicating that the disrupted mUG locus is inherited in a Mendelian fashion and that UG<sup>+/+</sup>, UG<sup>+/-</sup>, and UG<sup>-/-</sup> mice

ere equally viable at birth. However, UG<sup>-/-</sup> mice exhibited a novel phenotype in which they developed a progressive illness characterized by cachexia, heavy proteinuria, and hypocalcemia associated with profound weight loss. Proteinuria is a condition in which abnormally high levels of albumin and other serum proteins are excreted in the urine. It is indicative of glomerular dysfunction and renal failure. Histopathological examination of the kidneys of affected animals (as described above for lungs) revealed the fulminant renal glomerular disease shown in Figure 3. Compared with the glomeruli of the UG<sup>+/+</sup> mice, those of UG<sup>-/-</sup> mice were hypocellular and had massive eosinophilic proteinaceous deposits. The time course of the fatal renal disease in UG<sup>-/-</sup> mice was either early onset (4-5 week period) or late onset (10 month period). Those UG<sup>-/-</sup> mice that initially appeared healthy at 4 weeks of age had focal glomerular deposits at two months of age. At about 10 months, these mice had extreme cachexia similar to that of the mice dying of early onset disease. Heterozygotes had a milder form of the renal disease observed in UG<sup>-/-</sup> mice.

Histopathology of the kidneys of mice with late onset disease showed not only severe glomerulopathy as in the early onset disease, but also had marked fibrosis of the renal parenchyma and tubular hyperplasia (see Figure 3). Although the predominant pathology in the UG<sup>-/-</sup> mice was found in the kidneys, histopathological studies also uncovered occasional focal areas of necrosis in the pancreas which appeared to be vascular oriented. Moreover, focal areas in the thymus and in the spleen structures suggestive of apoptotic bodies were also found. Interestingly, the pancreas expresses the mUG gene, and this organ is also a rich source of group-I extracellular PLA<sub>2</sub>; since this is primarily a digestive enzyme, its activation may cause tissue injury.

Because uteroglobin proteins have been reported to have immunomodulatory and anti-inflammatory properties and because reactive amyloidosis is known to occur in response to inflammation, it was likely that the glomerular deposits in the mUG-null mice were amyloid proteins. Reactive amyloidosis is characterized by the deposition of amyloid protein and immune complexes. The identity of the renal deposits in the UG<sup>-/-</sup> mice was established by immunohistochemistry of kidney sections. Kidney sections from UG<sup>-/-</sup> and UG<sup>+/+</sup>

mice were stained with Congo red and examined under the polarized light. Amyloid proteins yield a positive birefringence in this test; however, the glomeruli of UG<sup>-</sup> mice were clearly negative. Immunofluorescence studies for the presence of IgA, IgG or IgM-immunocomplexes in the glomeruli of UG<sup>-</sup> mice and immunohistochemical analyses for the presence of major amyloid proteins were also negative. Thus, the glomerular deposits of UG<sup>-</sup> mice contained neither amyloid proteins nor immunocomplexes, and therefore, do not appear to be the result of an inflammatory response.

#### 10 **Example 6: Detection of Fn and Collagen in UG<sup>-</sup> kidneys**

The kidney deposits of UG<sup>-</sup> mice were examined by transmission electron microscopy to elucidate their structure and morphology. A kidney from a UG<sup>-</sup> mouse, with glomerular lesion, was fixed in formalin and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate for examination under the electron microscope. Photomicrographs were taken either at 6000X or at 60,000X. The deposits contained primarily two types of fibrillar structures: one type of long and striated fibrils which are relatively infrequent, the other short and diffuse which are more abundant (Figures 3E and 3F). Because ECM proteins, such as collagen and fibronectin, produce similar fibrillar structures, the glomerular deposits in UG<sup>-</sup> mice may contain these proteins.

The glomerular deposits were next analyzed by immunofluorescence using anti-mFn antibody. Formalin-fixed tissue sections were used for immunofluorescence using a rabbit anti-mFn and FITC-conjugated goat anti-rabbit IgG. Similarly, immunofluorescence studies using antibodies specific for mFn, collagen I and III, vitronectin, laminin and osteopontin were also done. Epifluorescence was photographed using a Zeiss Axiophot microscope. Fn-specific immunofluorescence in the renal glomeruli of wild-type mice was virtually undetectable (Figure 3G), that in the glomeruli of UG<sup>-</sup> littermates was intense (Figure 3H). When Masson's trichrome staining was used, the glomeruli of UG<sup>+/+</sup> mice were negative (Figure 3I) and those of UG<sup>-</sup> (Figure 3J) mice were positive, suggesting the presence of collagen



in the glomerular deposits. Immunofluorescence, using collagen I and collagen III-specific antibodies confirmed these results. Because Fn is known to interact with other extracellular matrix (ECM) proteins, we also tested for the presence of laminin, vitronectin and osteopontin in the glomeruli of UG<sup>+/+</sup> and UG<sup>-/-</sup> mice by  
5 immunohistochemistry, the results of which were negative.

**Example 7: Kidneys of UG<sup>-/-</sup> mice do not overproduce Fn**

In order to determine whether excessive production of Fn may account for its deposition in the renal glomeruli, we assessed the relative amount of Fn-mRNA in  
10 the kidneys, lungs, and the liver of UG<sup>-/-</sup> and UG<sup>+/+</sup> mice by RT-PCR and densitometry. The results indicate that relative amounts of Fn-mRNA were essentially identical in both UG<sup>+/+</sup> and UG<sup>-/-</sup> animals. Thus, over-production of Fn-mRNA was not a likely cause of Fn-deposition in the glomeruli of UG<sup>-/-</sup> mice. We then compared the Fn-protein in the plasma, kidneys, and the liver of UG<sup>-/-</sup> and UG<sup>+/+</sup>  
15 mice by SDS-PAGE under reducing conditions, and Western blotting. In the plasma, kidneys and the liver of wild-type mice only 220-kD Fn species could be detected; however, whereas the plasma and the liver lysate of UG<sup>-/-</sup> mice had the 220-kD Fn band, the kidney lysates contained another distinct, covalently linked, multimeric Fn-band (Figure 4A).

20

**Example 8: Elevated serum PLA<sub>2</sub> activity in UG<sup>-/-</sup> mice**

Based upon current concepts, critical initial steps in Fn matrix-assembly and fibrilogenesis, at least on the cell surface, are thought to involve integrin activation and Fn self-aggregation. Because UG is a potent inhibitor of soluble phospholipase  
25 A<sub>2</sub> (sPLA<sub>2</sub>), a key enzyme in the inflammatory pathway, the lack of mUG in UG<sup>-/-</sup> mice may contribute to the development of glomerulonephritis, an inflammatory renal disease. Thus, we measured PLA<sub>2</sub> activity in the serum of age, sex and weight-matched UG<sup>+/+</sup> (n=3) and UG<sup>-/-</sup> mice (n=3). The animals were sacrificed and serum PLA<sub>2</sub> activities of each sample were measured in triplicate using a PLA<sub>2</sub>-assay kit  
30 (Caymen Chemical) according to the instructions of the manufacturer. Protein

concentrations in the sera were determined by Bradford assay (Bio Rad) and specific activities of PLA<sub>2</sub> were calculated. The specific activities ( $\mu\text{mol}/\text{min}/\text{mg}$  protein) of serum PLA<sub>2</sub> of UG<sup>-/-</sup> mice [36 + 3.3 (SEM)] were significantly higher ( $p < 0.05$ ) than those of UG<sup>+/+</sup> mice [18 + 2.8 (SEM)]. These results raised the possibility that higher PLA<sub>2</sub> activity may lead to increased lysophosphatidic acid (LPA) production and consequently promote integrin activation and Fn-self aggregation in UG<sup>-/-</sup> mice.

**Example 9: Interaction of uteroglobin and fibronectin *in vitro***

To further understand how uteroglobin may prevent Fn self assembly, the ability of rhUG to disrupt mFn-Fn interaction *in vitro* was determined. Equimolar concentrations of rhUG and mFn were incubated to allow any protein binding or other interactions, then immunoprecipitated with anti-Fn-antibody, and the immunoprecipitates were resolved by SDS-PAGE under reducing conditions. Western blotting, as previously described, with either mFn or mUG antibody detected each protein, respectively. The results show that fibronectin co-immunoprecipitated with rhUG (Figure 4B). To confirm these results, the <sup>125</sup>I-rhUG was incubated with mFn and the complexes resolved by electrophoresis, using a 6% polyacrylamide gel under non-denaturing and non-reducing conditions (Figure 4C). Detection of a Fn-UG heteromer in the autoradiogram (lane 2) showed that soluble Fn interacts with UG *in vitro*. To ascertain whether Fn-UG heteromerization takes place *in vivo*, plasma of UG<sup>+/+</sup> and UG<sup>-/-</sup> mice was immunoprecipitated with an anti-mFn antibody that does not crossreact with rhUG (Figure 4D). Anti-mFn antibody co-precipitated both mFn and rhUG from the plasma of UG<sup>+/+</sup>, but not from UG<sup>-/-</sup> mice, suggesting that Fn-UG heteromers are present in the plasma of UG<sup>+/+</sup> mice. Therefore, the Fn-UG complex is not simply an artifact formed *in vitro* but occurs naturally in the serum.

To determine the specificity and affinity of UG binding to Fn, we incubated <sup>125</sup>I-Fn with unlabeled Fn in the presence and absence of UG. Any complexes were affinity-crosslinked with disuccinimidyl suberate (DSS). Using 24-well plates coated with human Fn (hFn) (Collaborative Biomedical Products), 3  $\mu\text{l}$  of <sup>125</sup>I-Fn (Sp. Act. 6 mCi/mg; ICN Biomedicals) was incubated in the absence and presence of either UG

or Fn ( $10^{-12}$ - $10^{-6}$  M) in 500  $\mu$ l HBSS at room temperature for 2 hr. SDS-PAGE and Western blotting of all Fn with UG antibody failed to detect any UG contamination. The radiolabeled complex was washed twice with PBS, solubilized in 1 N NaOH, neutralized with 1 N HCl, and radioactivity was measured by a gamma counter. In a separate experiment  $^{125}$ I-hFn (3  $\mu$ l) was incubated with 20  $\mu$ l (1 mg/ml) of mouse Fn in 40  $\mu$ l of HBSS, pH 7.6 in the absence or presence of increasing concentrations of reduced rhUG (5-500  $\mu$ g) at room temperature for 2 hours. The samples were crosslinked with 0.20 mM DSS at room temperature for 20 min., boiled in SDS-sample buffer for 5 min., electrophoresed on 4-20% SDS-polyacrylamide gel and autoradiographed. In the absence of UG,  $^{125}$ I-Fn formed a high molecular weight, radioactive complex with unlabeled Fn, but in the presence of UG the formation of Fn-Fn aggregates was inhibited in a manner dependent upon the UG concentration (Figure 4E).

To determine whether there is any difference between the binding affinities of Fn for UG and that of Fn for itself binding experiments were performed in which  $^{125}$ I-Fn was incubated with unlabeled Fn and immobilized on multiwell plates together with varying concentrations of UG. In separate experiments, binding studies of  $^{125}$ I-Fn with unlabeled, immobilized Fn using various concentrations of unlabeled soluble Fn, were also done. The Scatchard analyses of the data from both types of binding experiments yielded straight lines with dissociation constants (kds) of 13 nM for UG binding to Fn and 176 nM for Fn binding to itself. These results suggest that, due to a relatively higher binding-affinity of UG for Fn, UG effectively counteracts Fn self-aggregation. Affinity-crosslinking experiments in which radio-iodinated ( $^{125}$ I)-collagen I was incubated with unlabeled Fn in the absence or presence of UG, were also done as described above for Fn. Fifteen  $\mu$ l of either denatured or non-denatured  $^{125}$ I-collagen I (Sp. Act. 65.4 mCi/mg) were incubated with Fn in presence or absence of reduced UG (250  $\mu$ g), affinity crosslinked, electrophoresed and autoradiographed. The results indicate that UG counteracts the formation of high molecular weight  $^{125}$ I-collagen-Fn aggregates (Figure 4F).

**Example 10: *In vivo* inhibition of glomerular hFn deposition by rhUG**

To test whether rhUG protects the renal glomeruli from Fn accumulation, soluble human Fn (hFn) alone, or hFn mixed with equimolar concentrations of rhUG, was administered intravenously to UG<sup>+/+</sup> and to apparently healthy UG<sup>-/-</sup> littermates.

5 Human Fn (500  $\mu$ g/150  $\mu$ l PBS) was administered in the tail vein of two-month old, approximately 22 g, UG<sup>+/+</sup> and apparently healthy, UG<sup>-/-</sup> mice. Similarly, the control mice were injected with a mixture of 500  $\mu$ g of hFn either with equimolar concentrations of rhUG or albumin in 150  $\mu$ l PBS. Twenty-four hours after the last injection, the mice were sacrificed and various organs were fixed in buffered  
10 formalin. The histological sections of the kidneys and other organs were examined by immunofluorescence with a monospecific anti-hFn antibody (GIBCO BRL; clone 1) and FITC conjugated rabbit anti-mouse IgG (Cappel). In a separate experiment, UG<sup>+/+</sup> mice were injected with 1 mg of hFn alone in 150  $\mu$ l PBS daily for 3 consecutive days.

15 The rationale for injecting human Fn was to be able to discriminate between endogenous murine Fn and the administered hFn. The method of intravenous administration and immunohistochemical detection of hFn in various tissues have been described.

Human Fn immunofluorescence in the glomeruli of wild-type UG<sup>+/+</sup> mice  
20 injected with either a mixture of hFn and rhUG (1:1 molar ratio) or with hFn alone was similar (Figures 5A and 5B). However, the UG<sup>-/-</sup> mice injected with a mixture of hFn and UG showed little hFn-specific immunofluorescence in the glomeruli (Figure 5C), while those receiving Fn alone exhibited higher intensity immunofluorescence (Figure 5D). Administration of a mixture of hFn and BSA, as  
25 a control, yielded no protective effect.

To determine whether this UG protective effect could be overcome by injecting larger quantities of Fn in UG<sup>+/+</sup> mice, we injected 1 mg of hFn per animal daily for three consecutive days. Although intravenous administration of hFn to UG<sup>+/+</sup> mice at lower doses (500  $\mu$ g/animal) was not effective in causing any  
30 appreciable glomerular deposition (Figure 5A), the administration of higher doses (3

mg/animal) led to a significant accumulation. Thus, UG prevents glomerular Fn-deposition, and UG<sup>+/+</sup> as opposed to UG<sup>-/-</sup> mice have a higher threshold for the accumulation of soluble Fn, due to the presence of endogenous UG.

**5 Example 11: Inhibition of fibrillogenesis and Fn matrix assembly by rhUG in tissue culture cells**

To determine whether UG prevents Fn-fibrillogenesis and matrix assembly in a typical *in vitro* tissue culture assay, mouse embryonic fibroblasts were cultured  
10 in medium containing either soluble hFn alone or a mixture of equimolar concentrations of hFn and rhUG. Fn matrix assembly and fibrillogenesis in cultured cells (CRL6336, ATCC) were determined as described. The level of fibrillogenesis seen in the cells of cultures treated with hFn alone was much higher (Figure 5E) compared to those which received a mixture of hFn and rhUG (Figure 5F).

15

**Example 12: Detection of UG-Fn complexes in clinical samples**

Detection of UG-Fn complexes in clinical samples of bodily fluids such as serum, BAL fluids, and sputum is important in determining the role of this complex in human disease. A solution phase diagnostic assay for the detection of UG-Fn  
20 complexes is developed and the assay format is shown in Figure 6. The capture antibody, covalently linked to a solid support, is a monospecific rabbit polyclonal raised against the human protein. The solid support may be a bead, such as a magnetic bead, a tube, or an ELISA plate. The solid support affords the flexibility of performing wash steps after each binding reaction in order to obtain more  
25 consistent results with a variety of sample types. The detection antibody is specific for Fn, and available from a number of commercial sources. An anti-IgG antibody, conjugated to an enzyme such as horse radish peroxidase (HRP), is then used to detect the anti-Fn IgG at the end of the molecular chain in a standard enzymatic reaction in which the enzyme substrate is converted to a chromogenic or fluorogenic  
30 compound that is quantitated with a spectrophotometer or fluorimeter (Amersham).

The detection limit for this assay is 500  $\mu\text{g}$  of UG-Fn complex per ml of sample fluid.

### Example 13: Uteroglobulin Deficiencies

5        A transient but acute deficiency of hUG is created by the blood-cleansing technique known as clinical dialysis, including hemodialysis, peritoneal dialysis and continuous dialysis (CRRT). All forms of clinical dialysis involve the use of a semi-permeable membrane to filter toxic bodily waste products, including chemical metabolites such as urea, and small proteins such as beta2-microglobulin, out of the  
10    blood.

UG is an extremely compact protein, known for its anomolous migration in SDS-PAGE, corresponding to a molecular weight of approximately 10-13 kDa, despite its true molecular weight of 15.7 kDa. Therefore, the UG dimer was expected to behave as a 10-13 kDa protein in dialysis experiments. Surprisingly, it  
15    was found that the dimer is so compact that it passed through an 8.0 kDa MWCO dialysis membrane. UG also passed through a 14.0 kDa MWCO dialysis membrane.

The composition of the dialysis membranes used in these examples are similar, if not identical, to the composition of the majority of membranes manufactured and used for clinical dialysis. They consist of regenerated cellulose or cellulose acetate.

20        For this experiment, 1.0 ml aliquots of two partially purified (one >90% pure and one approximately 70% pure) rhUG cell lysates, with no buffer additives, were dialyzed against 1000 mls of unbuffered 50 mM ammonium acetate, using three sizes of dialysis tubing: 3.5 kDa, 8.0 kDa, and 14.0 kDa (Spectra/Por; Thomas Scientific). There were four changes of buffer for each sample over a 48 hour time  
25    period, all done at room temperature (about 25-27°C). The appearance of each dialysis sample changed from a clear yellow to a clear, colorless liquid. Dialysis tubing was checked for leaks at the beginning and end of the process by brief application of pressure directly to the tubing (squeezing) and observation of any leaks, of which there were none. Tubing was double clamped at either end to further insure  
30    against leaks.

Figure 7 shows the SDS-PAGE analysis of these results. The 90% pure pre-dialysis sample is shown in lane 7 and 8 next to the three post-dialysis samples in lanes 1, 2, and 3. The UG dimer is no longer present in the lanes representing the samples dialysed with 8.0 kDa MWCO membranes. These results were later  
5 confirmed with different batches of partially purified UG preparations.

While the invention has been described in connection with what is presently considered to be the most practical and preferred embodiments, it is to be understood that the invention is not to be limited to the disclosed embodiments but, on the  
10 contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

REFERENCES

1. Levin, S.W. *et al.*, Life Sci. 38: 1813-1819 (1986);
2. Singh G. *et al.*, Biochem. Biophys. Acta. 1039: 348-355(1990);
3. Mantile, G. *et al.*, J. Biol. Chem 268: 20343-20351 (1993);
- 5 4. Singh, G. *et al.*, J. Histochem. Cytochem. 36: 73-80 (1987);
5. Bernard, A. *et al.*, Clin. Chem. 38: 434-435 (1992);
6. Dhanireddy, R. *et al.*, Pediatric Res. 23: 463A (1988);
- 10 7. Dhanireddy, R. *et al.*, Pediatric Res. 33: 323A (1993);
8. Piomelli, D., Op. In Cell Biol. 5: 274-280(1993);
9. Krishnan, R.S. *et al.*, Science 158: 490-492 (1967);
- 15 10. Beier, H. Verhandl Deut. Zool. Ges. Heidelberg (1968);
11. Umland, T.C. *et al.*, Nature Stuct. Biol. 1: 538-545 (1994);
- 20 12. Hard, T. *et al.*, Nature. Struct. Biol. 2: 938-989 (1995);
13. Umland, T.C. *et al.*, Nature Struct. Biol. 2: 919-922(1995);
14. Stripp, B. R. *et al.*, Am. T. Physio. 271 (Lung Cell. Mol. Physiol. 15):  
25 L656-L664 (1996);
15. Lesur, O. *et al.*, Am. T. Respir. Crit. Care Med. 152: 290-297 (1995);
16. Glaser, K.B., Adv. Pharmacol. 32: 31-66 (1995);
- 30 17. Tykka, H.T. *et al.*, Scand. J. Gastroenterol. 20: 5-12 (1985);
18. Sheuer, W., Klin. Wochenschr. 67: 153-159 (1989);
- 35 19. Barnes, H.J. *et al.*, J. Mol. Biol., Feb. 23, 1996;
20. Aoki, A. *et al.*, Mol. Hum. Reprod. 2: 419-497 (1996);
21. Anderson and Kurkland, Microbiological Reviews 54: 198-210 (1990);  
40



22. Miele, L. *et al.*, J. Biol. Chem. 265: 6427-6435 (1990);
23. Coalson, J.J. *et al.*, Exp. Mol. Pathol. 37: 355-360 (1982);
- 5 24. Nagy, A. *et al.*, Proc. Natl. Acad. Sci. 90: 8424 (1993);
25. Capecchi, M.R., Science, 244: 1288 (1989);
- 10 26. Harlow, E. and Lane D. Antibodies: A Laboratory Manuel, 1st Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988;
27. Mantile, G. *et al.*, J. Biol. Chem. 267: 20343 (1993);
28. Ruoslahti, E. Ann Rev. Biochem. 57: 375 (1988);
- 15 29. R.O. Hynes, Fibronectins, New York: Springer-Verlag (1990);
30. Chernousor, M.A. *et al.*, J. Biol. Chem. 266: 10857 (1991);
- 20 31. Zhang, Q. *et al.*, J. Cell. Biol. 127: 1447 (1994);
32. Wu, C. *et al.* Cell 83: 715 (1995);
33. Zhang, Q. *et al.*, J. Biol. Chem. 271: 33284 (1996);
- 25 34. Border, W.A. *et al.*, J. Clin. Invest. 90: 1 (1992);
35. Peri, A., *et al.*, J. Clin. Invest. 92: 2099 (1995);
- 30 36. Peri, A. *et al.*, J. Clin. Invest. 96: 343 (1995);
37. Oh, E. *et al.*, Proc. Natl. Acad. Sci. (USA) 78: 3218 (1981);
38. Mosher, D.F. *et al.*, Curr. Biol. 4: 810 (1992).

**WHAT IS CLAIMED IS:**

1. A pharmaceutical composition comprising a PLA<sub>2</sub> inhibiting effective  
5 amount of recombinant human uteroglobin (rhUG) or a fragment or a derivative thereof and a pharmaceutically acceptable carrier or diluent.
2. The pharmaceutical composition of claim 1 further comprising a lung surfactant.
3. The pharmaceutical composition of claim 1 wherein said rhUG has a  
10 purity of about 75% to about 100%.
4. The pharmaceutical composition of claim 1 wherein said rhUG has a purity of about 90% to about 100%.
5. The pharmaceutical composition of claim 1 wherein said rhUG has a purity of at least 95%.
- 15 6. A pharmaceutical composition comprising a fibronectin binding effective amount of recombinant human uteroglobin (rhUG) or a fragment or a derivative thereof and a pharmaceutically acceptable carrier or diluent.
7. The pharmaceutical composition of claim 6 further comprising a lung surfactant.
- 20 8. The pharmaceutical composition of claim 6 wherein said rhUG has a purity of about 75% to about 100%.
9. The pharmaceutical composition of claim 6 wherein said rhUG has a purity of about 90% to about 100%.
10. The pharmaceutical composition of claim 6 wherein said rhUG has a  
25 purity of at least 95%.
11. A pharmaceutical composition comprising a PLA<sub>2</sub> inhibiting or fibronectin binding effective amount of UG in admixture with an active agent for treatment of a target indication.
12. The pharmaceutical composition of claim 11, wherein said PLA<sub>2</sub>  
30 inhibiting or fibronectin binding effective amount of rhUG reduces inflammation and

thereby insures that an effective amount of said active agent reaches the treatment site.

13. A method for inhibiting PLA<sub>2</sub> enzymes *in vivo* in a mammal in need of such treatment, said method comprising administering to said mammal a PLA<sub>2</sub> inhibiting effective amount of native or recombinant human uteroglobin (UG) or a fragment or derivative thereof.

14. The method of claim 13 wherein said UG has a purity of between about 75% to about 100%.

15. The method of claim 13 wherein said UG has a purity of between about 90% to about 100%.

16. The method of claim 13 wherein said UG has a purity of at least 95%.

17. The method of claim 13 wherein said UG is recombinant (rhUG), having substantially the same sequence as native human uteroglobin.

18. The method of claim 13 wherein said PLA<sub>2</sub> inhibiting effective amount of UG is 20ng/kg to 500 mg/kg.

19. The method of claim 13 wherein said UG is administered in association with a lung surfactant.

20. The method of claim 19 wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

21. The method of claim 13 wherein said UG is administered by the endotracheal, ophthalmic, intravenous, systemic, intraperitoneal, intramuscular, or oral routes.

22. The method of claim 13 wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.

23. The method of claim 13 wherein said method is for the treatment of a condition selected from the group consisting of systemic inflammation, asthma, cystic fibrosis, ocular inflammation, premature labor, infertility, rheumatoid arthritis, type 1 diabetes, nephropathy, inflammatory bowel disease, Crohn's disease, ulcerative colitis, pancreatitis, peritonitis, allergy, multiple organ failure, ARDS, acute renal

failure, inflammation secondary to infection or surgery, and transplanted organ rejection.

24. The method of claims 23 wherein ocular inflammation results from autoimmune uveitis and corneal transplant surgery.

5 25. The method of claim 13 wherein said method is for the treatment of inflammation and immune modulation.

26. A method for treating or preventing an inflammatory condition in a patient in need of such treatment, said method comprising administering to said patient an anti-inflammatory effective amount of native or recombinant UG or a  
10 fragment or derivative thereof.

27. The method of claim 26 wherein said UG has a purity of between about 75% to about 100%.

28. The method of claim 26 wherein said UG has a purity of between about 90% to about 100%.

15 29. The method of claim 26 wherein said UG has a purity of at least 95%.

30. The method of claim 26 wherein said UG is recombinant (rhUG) and has substantially the same sequence as the native human uteroglobin.

31. The method of claim 26 wherein said  $\text{PLA}_2$  inhibiting effective amount of UG is 20ng/kg to 500 mg/kg.

20 32. The method of claim 26 wherein said UG is administered in association with a lung surfactant.

33. The method of claim 32 wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

34. The method of claim 26 wherein said UG is administered by the  
25 endotracheal, ophthalmic, intravenous, systemic, intraperitoneal, intramuscular, or oral routes.

35. The method of claim 26, wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.

36. The method of claim 26, wherein said inflammatory condition is  
30 neonatal RDS.

37. The method of claim 26 wherein said inflammatory condition is adult RDS.

38. The method of claim 26, wherein said inflammatory condition is caused by a viral infection, bacterial infection, parasitic infection, or fungal infection.

5        39. The method of claim 26, wherein said method is for the treatment of an inflammatory condition selected from the group consisting of systemic inflammation, asthma, cystic fibrosis, ocular inflammation, premature labor, remote organ failure, rheumatoid arthritis, pancreatitis, septic shock, collagen vascular disease, anaphylactic shock, trauma-induced shock, acute renal failure, cystitis, 10 inflammation of the urethra, inflammation of the ureter, bladder inflammation, interstitial cystitis, vaginitis, inflamed cervix, pelvic inflammatory disease, inflammation of the ovariosalpingitis, inflammation of the fallopian tubes, penile inflammation, prostate inflammation, inflammation of seminal tubules and seminal vesicles, testicular inflammation, inflammation of vas deferens, epididymis, and 15 prostate gland, retinitis, burn damage due to fire or chemical burn, bronchial asthma, ARDS, neonatal RDS, directiculitis, neonatal necrotizing enterocolitis, stomach ulcers, gastrointestinal reflux disease, tonsillitis, hemorrhoids, otitis media, psoriasis, hives, allergic dermatitis, contact dermatitis, chemical dermatitis, endocarditis, and infertility.

20        40. The method of claim 39, wherein said inflammation is ocular inflammation that results from autoimmune uveitis, CMV retinitis, bacterial infection, viral infection, parasitic infection, the presence of an infectious agent, retinoblastoma, radiation exposure, allergic response, and corneal transplant surgery.

41. A method for treating or preventing a fibrotic condition in a patient in 25 need of such treatment, said method comprising the step of administering to a patient a fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

42. The method of claim 41, wherein said UG has a purity of between about 75% to about 100%.

43. The method of claim 41, wherein said UG has a purity of between about 90% to about 100%.

44. The method of claim 41, wherein said UG has a purity of at least 95%.

45. The method of claim 41, wherein said UG is recombinant (rhUG) and  
5 has substantially the same sequence as native human uteroglobin.

46. The method of claim 41, wherein said fibronectin binding effective amount of UG is 20ng/kg to 500 mg/kg.

47. The method of claim 41, wherein said UG is administered in association with a lung surfactant.

10 48. The method of claim 47, wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

49. The method of claim 41, wherein said UG is administered by the endotracheal, ophthalmic, intravenous, intraperitoneal, intramuscular, liquid or oral routes.

15 50. The method of claim 41, wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.

51. The method of claim 41, wherein said fibrotic condition is pulmonary fibrosis.

52. The method of claim 41, wherein said fibrotic condition is renal  
20 fibrosis.

53. The method of claim 41, wherein said fibrotic condition is vascular fibrosis.

54. A method for treating or preventing an inflammatory or fibrotic condition characterized by a deficiency of endogenous UG, said method comprising  
25 administering to a patient in need of such treatment a compensating amount of native or recombinant UG or a fragment or derivative thereof.

55. The method of claim 54, wherein said UG has a purity of between about 75% to about 100%.

56. The method of claim 54, wherein said UG has a purity of between  
30 about 90% to about 100%.

57. The method of claim 54, wherein said UG has a purity of at least 95%.

58. The method of claim 54, wherein said UG is recombinant (rhUG) and has substantially the same sequence as native human uteroglobin.

59. The method of claim 54, wherein said compensating amount UG is  
5 20ng/kg to 500 mg/kg.

60. The method of claim 54, wherein said UG is administered in association with a lung surfactant.

61. The method of claim 60, wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

10 62. The method of claim 54, wherein said UG is administered by the endotracheal, ophthalmic, intravenous, systemic, intraperitoneal, intramuscular, or oral routes.

63. The method of claim 54, wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.

15 64. The method of claim 54, wherein said method is for the treatment of an inflammatory or fibrotic condition characterized by a deficiency of endogenous UG, wherein said condition is selected from the group consisting of neonatal broncho-pulmonary dysplasia, complications of hemodialysis, bleomycin lung chronic obstructive pulmonary disease, and inherited glomerulopathy.

20 65. The method of claim 26, wherein UG is administered in combination with an active agent selected from the group consisting of steroids, nonsteroidal antiinflammatory agents, chemotherapeutics, analgesics, antibodies, antiparasites, antivirals, antibiotics, antifungals, vaccines, tumor vaccines, immunosuppressives, hematopoietic growth factors, antithrombotics, cardiovascular drugs, one or more  
25 vitamin and mineral supplements, and fertility drugs.

66. The method of claim 44, wherein UG is administered in combination with an active agent selected from the group consisting of steroids, nonsteroidal antiinflammatory agents, chemotherapeutics, analgesics, antibodies, antiparasites, antivirals, antibiotics, antifungals, vaccines, tumor vaccines, immunosuppressives,

growth factors, antithrombotics, cardiovascular drugs, one or more vitamin and mineral supplements, and fertility drugs.

67. The method of claim 54, wherein UG is administered in combination with an active agent selected from the group consisting of steroids, nonsteroidal  
5 antiinflammatory agents, chemotherapeutics, analgesics, antibodies, antiparasites, antivirals, antibiotics, antifungals, vaccines, tumor vaccines, immunosuppressives, growth factors, antithrombotics, cardiovascular drugs, one or more vitamin and mineral supplements, and fertility drugs.

68. A cosmetic composition comprising a  $PLA_2$  inhibiting effective amount  
10 of native or recombinant human uteroglobin (UG) or a fragment or derivative thereof and a pharmaceutically acceptable carrier or diluent.

69. The cosmetic composition of claim 68 further comprising a lung surfactant.

70. The cosmetic composition of claim 68 wherein said UG has a purity  
15 of about 75% to about 100%.

71. The cosmetic composition of claim 68 wherein said UG has a purity of about 90% to about 100%.

72. The cosmetic composition of claim 68 wherein said UG has a purity of at least 95%.

20 73. A cosmetic composition comprising a fibronectin binding effective amount of native or recombinant human uteroglobin (UG) or a fragment or derivative thereof, and an acceptable carrier or diluent.

74. The cosmetic composition of claim 73 further comprising a lung surfactant.

25 75. The cosmetic composition of claim 73 wherein said UG has a purity of about 75% to about 100%.

76. The cosmetic composition of claim 73 wherein said UG has a purity of about 90% to about 100%.

30 77. The cosmetic composition of claim 77 wherein said UG has a purity of at least 95%.



78. A blood supplement comprising a PLA<sub>2</sub> inhibiting effective amount of native or recombinant human uteroglobin (UG) or a derivative or fragment thereof, and a pharmaceutically acceptable carrier or diluent.

79. The blood supplement composition of claim 78 further comprising a  
5 lung surfactant.

80. The blood supplement composition of claim 78 wherein said UG has a purity of about 75% to about 100%.

81. The blood supplement composition of claim 78 wherein said UG has a purity of about 90% to about 100%.

10 82. The blood supplement composition of claim 78 wherein said UG has a purity of at least 95%.

83. A blood supplement composition comprising a fibronectin binding effective amount of native or recombinant human uteroglobin (UG) or a fragment or derivative thereof, and a pharmaceutically acceptable carrier or diluent.

15 84. The blood supplement composition of claim 83 further comprising a lung surfactant.

85. The blood supplement composition of claim 83 wherein said UG has a purity of about 75% to about 100%.

20 86. The blood supplement composition of claim 83 wherein said UG has a purity of about 90% to about 100%.

87. The blood supplement composition of claim 83 wherein said UG has a purity of at least 95%.

25 88. An assay for quantitating uteroglobin-fibronectin complexes in a clinical sample, wherein a clinical sample suspected of containing one or more uteroglobin-fibronectin complexes is

- (a) contacted with an antigen capture agent,
  - (b) an antigen detection agent is added to said sample, and
  - (c) the presence of any complex bound to said capture agent is detected using a secondary antibody conjugated to a moiety capable of being detected
- 30 by the addition of a compound which reacts with said chemical moiety.

89. The assay of claim 88 wherein said antigen capture agent is a monospecific rabbit polyclonal antibody, immobilized on an insoluble support.

90. The assay of claim 88 wherein the secondary antibody is an anti-IgG antibody conjugated to an enzyme and the compound which reacts with the chemical moiety is a substrate for the enzyme which is converted to a chromogenic or fluorogenic compound upon reaction with the enzyme.

91. The assay of claim 90 wherein the enzyme is horse radish peroxidase.

92. The assay of claim 88 wherein the antigen detection agent is an antibody specific for fibronectin.

93. A method for treating or preventing fibrillogenesis in a patient in need of such treatment, said method comprising the step of administering to a patient a fibronectin binding effective amount of native or recombinant UG or a fragment or derivative thereof.

94. The method of claim 93, wherein said UG has a purity of between about 75% to about 100%.

95. The method of claim 93, wherein said UG has a purity of between about 90% to about 100%.

96. The method of claim 93, wherein said UG has a purity of at least 95%.

97. The method of claim 93, wherein said UG is recombinant (rhUG) and has substantially the same sequence as native human uteroglobin.

98. The method of claim 97, wherein said fibronectin binding effective amount of UG is 20ng/kg to 500 mg/kg.

99. The method of claim 97, wherein said UG is administered in association with a lung surfactant.

100. The method of claim 99, wherein said lung surfactant is present in an amount of about 20% to about 80% by weight.

101. The method of claim 93, wherein said UG is administered by the endotracheal, ophthalmic, intravenous, intraperitoneal, intramuscular, liquid or oral routes.

102. The method of claim 93, wherein said UG is administered as a liquid or semi-aerosol via an intratracheal tube.
103. The method of claim 93, wherein said fibrotic condition is pulmonary fibrosis.
- 5 104. The method of claim 93, wherein said fibrotic condition is renal fibrosis.
105. The method of claim 93, wherein said fibrotic condition is vascular fibrosis.
106. The method of claim 38 wherein said viral infection is CMV retinitis.
- 10 107. The method of claim 38 wherein said bacterial infection is pneumonia or cystitis.
108. The method of claim 38 wherein said parasitic infection is schistosomiasis.
109. The method of claim 38 wherein said fungal infection is vaginal
- 15 candidiasis.

*FIG. 1*

Human CC10 kDa: 1 EICPSFQRVIETLLMDTPSSYEAAAMELSPDQDMREAGAKKLVDTLPQKPRESIIKLMEKIAQSSLCN 70  
 ICP F VIE LL TPSSYE F PC M AG Q KK D LPQ RE I KL EKI S LC  
 Rabbit Utg: GICPRFAHVIENTLLGTPSSYETSLKEFEPPDDTMKDAGMCKKVLDSLPTQTTRENIMKLTKEIVKSPLC

ICP F V E LL S YEAA F P D AG QLK LVDILPQ R I KL EKI S LC  
 Rat CC10: DICPGFLQVLEALLLGSESNYEAAALKPFNPASDLQNAQTQLKRLVDTLPQETRINIVKLTKEILTSPLCEQDLRV

ICP F V E LLM S Y A F P D AG QLK LVDILPQ R I KL EKI S LC  
 Mouse CC10: DICPGFLQVLEALLMESESGYVASLKPFNPGSDLQNAQTQLKRLVDTLPQETRINIMKLTKEILTSPLCKQDLRF

For comparison, amino acid residues are shown above the primary sequences correspond to residues found at the same position in the human protein. These sequences are predicted by translation of the nucleotide sequences deposited in the Genebank. These proteins are secreted proteins and the N-terminal signal sequences, that are translated and processed off, have been edited out for this comparison.

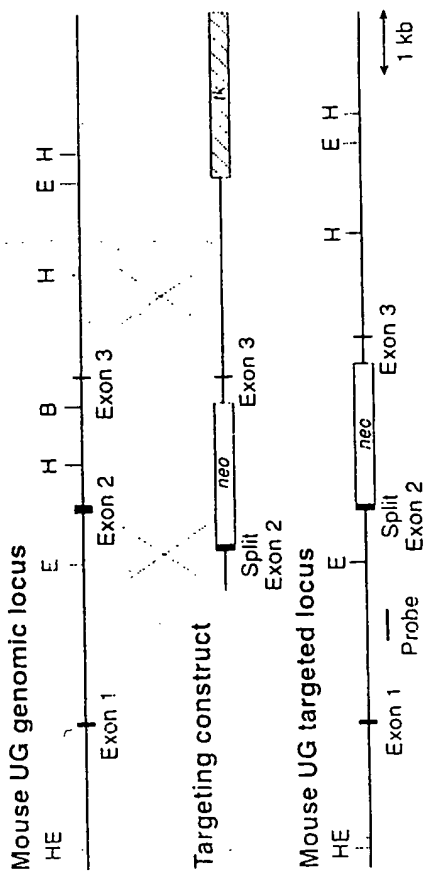


FIG. 2A

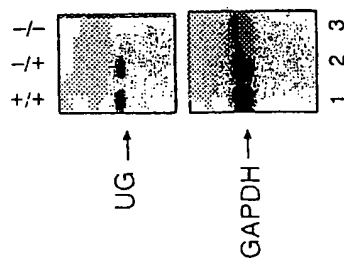


FIG. 2E

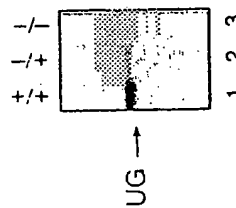


FIG. 2F

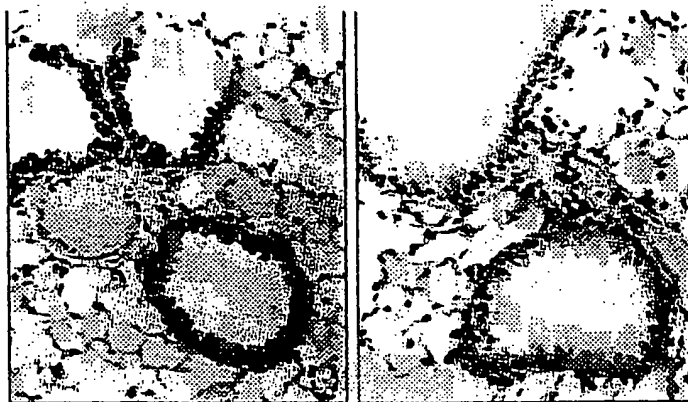


FIG. 2G

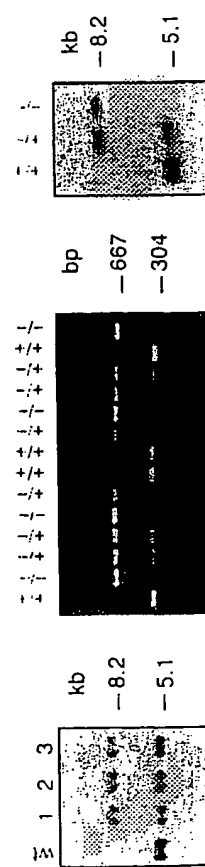


FIG. 2D

FIG. 2C

FIG. 2B



FIG. 3D

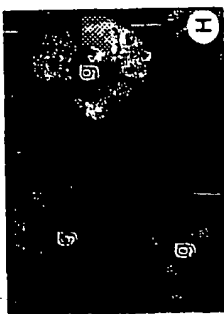


FIG. 3H



FIG. 3C

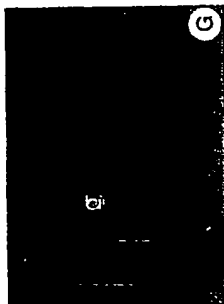


FIG. 3G



FIG. 3J



FIG. 3B



FIG. 3F



FIG. 3I



FIG. 3A



FIG. 3E

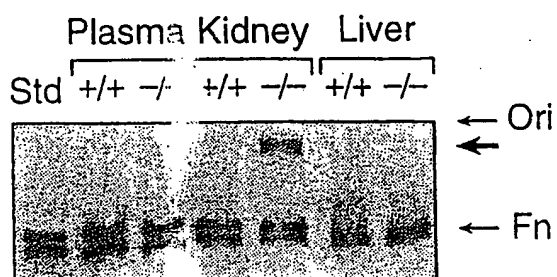


FIG. 4A

FIG. 4B

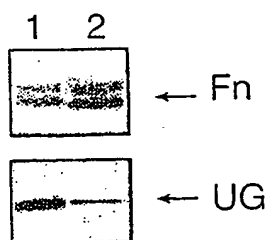


FIG. 4C

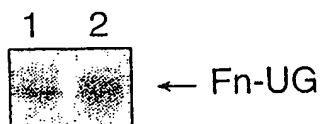


FIG. 4D

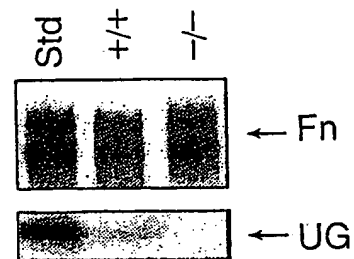


FIG. 4E

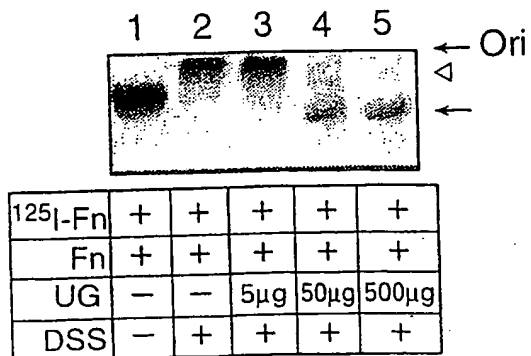
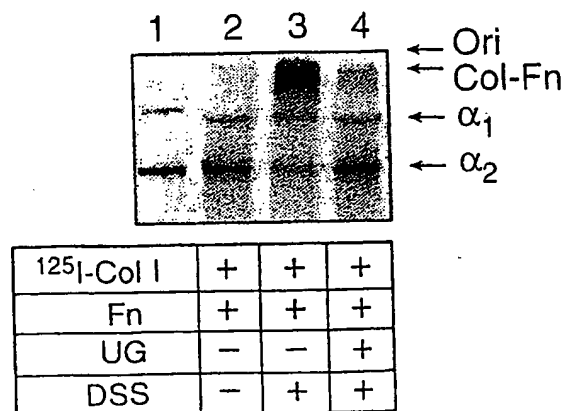
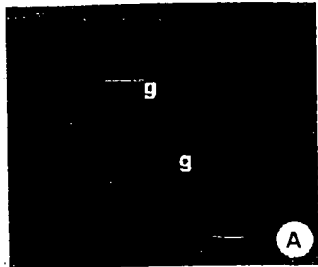
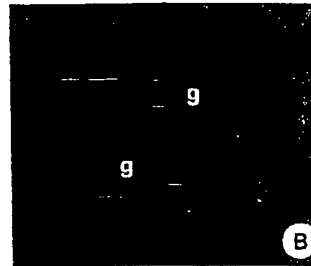


FIG. 4F

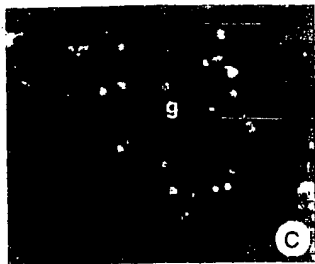




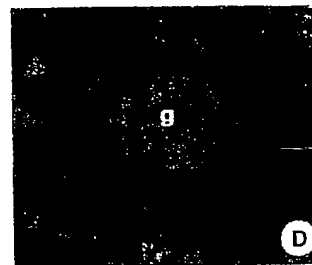
*FIG. 5A*



*FIG. 5B*



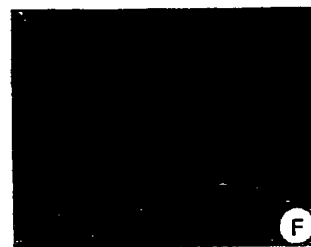
*FIG. 5C*



*FIG. 5D*

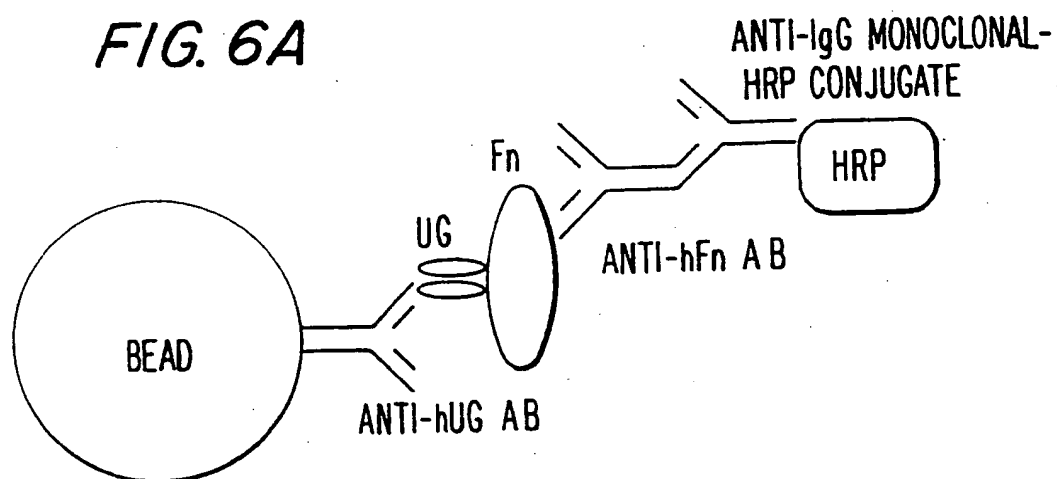
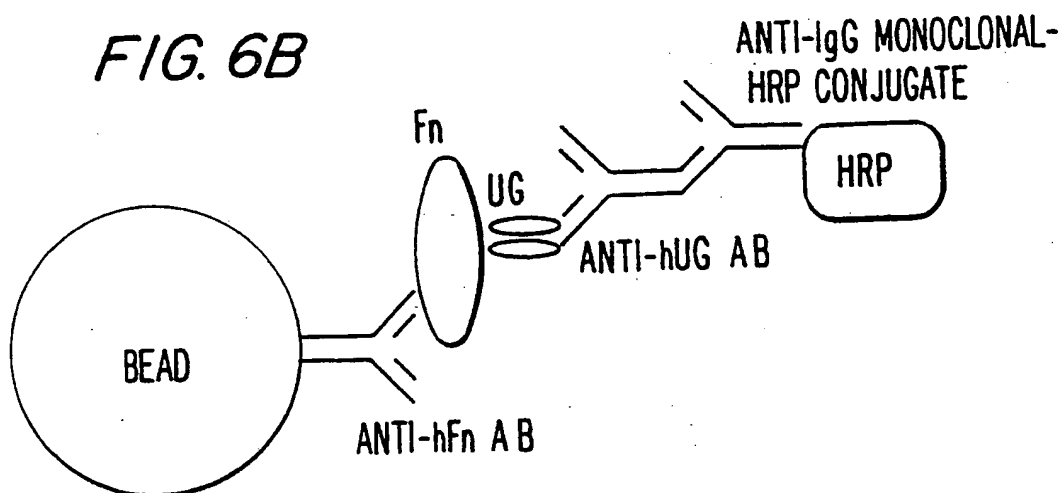


*FIG. 5E*



*FIG. 5F*



*FIG. 6A**FIG. 6B*

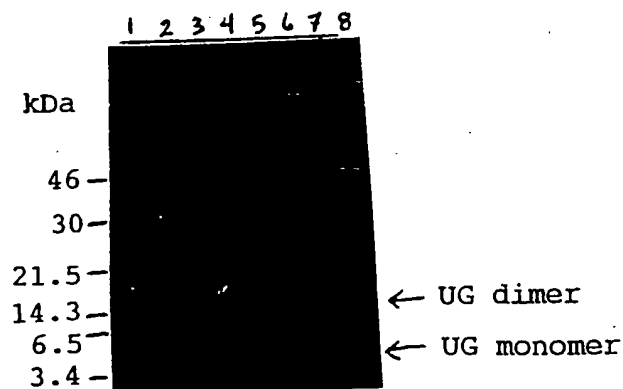


Figure 1: SDS-PAGE analysis of dialysis of uteroglobin and identification by combined reduction and denaturation. Lane 1 contains an ~95% pure rhUG sample dialyzed as described, using a 3.5 kDa MWCO membrane, representing the material retained within the dialysis tubing. Lane 3 contains an ~70% pure rhUG sample dialyzed as described, using a 3.5 kDa MWCO membrane, representing the material retained within the dialysis tubing. Lane 2 contains the same ~70% pure rhUG sample dialyzed using an 8.0 kDa MWCO membrane, representing the material retained within the dialysis tubing. Lane 4 contains the protein size marker. Lane 5 contains the pre-dialysis sample of lane 1 and lane 6 shows the same sample with a reducing agent, DTT, added to break the cysteine bonds between UG monomers. Lane 7 contains the pre-dialysis sample of lanes 2 and 3 and lane 8 contains the same sample plus DTT. Equal volumes of all lanes and samples (15 microliters) were used to facilitate comparisons.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11026

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) :A61K 38/17; C07K 14/875 US CL :514/12 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 514/12  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, CAPLUS search terms: uteroglobin, phospholipase, fibronectin, lung surfactant		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	LEYTON et al. Recombinant human uteroglobin inhibits the in vitro invasiveness of human metastatic prostate tumor cells and the release of arachidonic acid stimulated by fibroblast-conditioned medium. Cancer Res. 15 July 1994, Vol. 54, pages 3696-3699, see Abstract and page 3697, column 1, full paragraph 1.	1, 3-6, 8-10 -- 2, 7, 11, 12
Y	EDELSON et al. Acute lung injury induced by phospholipase A <sub>2</sub> : Structural and functional changes. Am. Rev. Respir. Dis. MAY 1991, Vol. 143, pages 1102-1109, see page 1102, column 1, full paragraph 1, paragraphs bridging columns 1-2 and columns 2-3, paragraph bridging pages 1105-1106.	2, 7
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* *A* *E* *L* *O* *P*	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	*T* *X* *Y* *G* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
Date of the actual completion of the international search 17 AUGUST 1998		Date of mailing of the international search report 24 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer DAVID S. ROMEO Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11026

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,266,562 A (MUKHERJEE et al.) 30 November 1993, column 8, full paragraph 1.	11, 12

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11026

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-12
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☒ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/11026

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-5, drawn to a pharmaceutical composition.

Group II, claim(s) 6-10, drawn to a pharmaceutical composition.

Group III, claim(s) 11-12, drawn to a pharmaceutical composition.

Group IV, claim(s) 13-25, drawn to a method of inhibiting  $PLA_2$  *in vivo*.

Group V, claim(s) 26-40, 65, and 106-109, drawn to a method of treating an inflammatory condition.

Group VI, claim(s) 41-53, and 66, drawn to a method of treating a fibrotic condition.

Group VII, claim(s) 54-64, and 67, drawn to a method of treating a uteroglobin deficiency.

Group VIII, claim(s) 68-72, drawn to a cosmetic composition.

Group IX, claim(s) 73-77, drawn to a cosmetic composition.

Group X, claim(s) 78-82, drawn to a blood supplement.

Group XI, claim(s) 83-87, drawn to a blood supplement.

Group XII, claim(s) 88-92, drawn to an assay for quantitating uteroglobin.

Group XIII, claim(s) 93-105, drawn to a method of treating fibrinolysis.

The inventions listed as Groups I-XIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

With respect to unity of invention PCT Rule 13.1 states:

The international application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention").

Additionally, PCT Rule 13.2 states:

Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

With regard to the application of PCT Rule 13, 37 CFR § 1.475 concerning unity of invention states:

(a) An international and a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in an application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

(c) If an application contains claims to more or less than one of the combinations of categories of invention set forth in paragraph (b) of this section, unity of invention might not be present.

(d) If multiple products, processes of manufacture or uses are claimed, the first invention of the category first mentioned in the claims of the application and the first recited invention of each of the other categories related

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11026

thereto will be considered as the main invention in the claims, see PCT Article 17(3)(a) and 37 C.F.R. § 1.476(c).

(e) The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim.

Groups I-XIII recite a plurality of pharmaceutical compositions and/or methods each having a different intended use. The single inventive concept joining these disparate intended uses appears to be that the pharmaceutical compositions comprise and the methods use recombinant human uteroglobin. In order for the inventions of groups I-XIII to have unity of invention it is necessary that the single inventive concept be a contribution to the prior art. However, LEYTON et al. (Cancer Research 54:3696-3699, July 15, 1994) teach recombinant human uteroglobin, teach that 1  $\mu$ M recombinant human uteroglobin inhibits arachidonic acid release, and suggest recombinant human uteroglobin inhibits tumor cell invasiveness through inhibition of PLA<sub>2</sub>-mediated arachidonic acid release. A pharmaceutical composition comprising a PLA<sub>2</sub> inhibiting effective amount of recombinant human uteroglobin would have been obvious to one of ordinary skill in the art. Therefore, the inventions of groups I-XIII do not fulfill the requirements for unity of invention with respect to a contribution which each of the inventions makes over the prior art. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record.**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**